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XX. STUDIES IN ANAEMIA. I.

THE INFLUENCE OF DIET ON THE OCCURRENCE OF SECONDARY ANAEMIA FOLLOWING REPEATED HAEMORRHAGES IN RATS.

By JAMES MATTHEWS DUNCAN SCOTT (*John Lucas Walker Student*).

From the Physiology Laboratory, Cambridge.

(*Received January 2nd, 1923.*)

Aims.

It seems a very obvious theorem that repeated haemorrhages would produce a chronic anaemia much more easily in an animal fed on a poor diet which lacks food factors essential for the formation of haemoglobin, such for instance as iron, than in one fed on a satisfactory and complete diet, and the first part of the following research was undertaken to demonstrate this. In practice several limitations of this principle are found. Thus one of the unpurified diets poorest in iron, white bread and whole milk, contains some materials for blood formation. Moreover there is a marked tendency even on a comparatively iron-poor diet to storage of a reserve of blood forming materials. Further, a limitation in the contrary direction is that a diet which is adequate in a fully grown animal to prevent an anaemia is inadequate in a similarly bled growing animal, presumably because it has not only to maintain its blood mass but also to increase it. These points are brought out in the following work.

Preliminary work on cats.

When this work was begun cats were chosen in preference to rats because it was easy to withdraw blood repeatedly from them by cardio-puncture under an anaesthetic. This avoided open operation and the animals were perfectly well in 24 hours. Five cats were fed on bread, milk and fat and were subjected every week to bleedings amounting each to 1% of the body weight. Controls were fed on the same diet but not subjected to bleeding. Other controls were given meat in addition to the bread and milk and were subjected to similar haemorrhages. The idea underlying the research was to find out if the first set of cats could be brought to a stage where they exhibited a chronic anaemia characterised by a fall in the colour index of the corpuscle. This was the case. The colour index of the cats fed on bread and milk and bled maintained itself or even increased at first, but then fell to sub-normal. The cats which were fed on bread and milk maintained their colour index. The controls which

were to be given meat and bled gave considerable trouble, as an infectious gastro-intestinal disease broke out amongst them and the mortality amongst them and the difficulty of stamping it out led eventually to my abandoning the use of these animals and substituting rats. The difficulty of bleeding rats repeatedly, which had previously been an obstacle to their use, was solved by having recourse to leeches, as will be described later.

Outline of work on rats.

Rats are suitable animals for this work, because so much research on diets has been done on them. They were bred from mothers which had been fed before and during pregnancy and weaning on a liberal mixed diet.

In the inception of the work unpurified diets were used, the basal diet being white bread and whole milk and the ration the ample one of 20 g. white bread and 75 cc. whole milk per 100 g. rat per day. In addition certain controls were given from the time of weaning a liberal supply of green stuff, cabbage, etc., to the amount of 10 g. per 100 g. rat per day. The actual amount of green food eaten is not known but it was certainly one-half and may have been as much as three-quarters of the green food given. The rats exhibited marked preference for the tender parts of plants, *e.g.* the heart leaves of cabbage. Thus the two diets differed only in the presence or absence of green stuff but the difference in the result might be assigned to one or more of several factors:

1. *Differences in iron content.* The results of different observers taken from Sherman [1907] are given in tabular form.

Bread.

Observer	Iron per 100 g. mgm.	
Stockman	0·6	Fine bread
„	0·4	Common bread
Sherman	0·7	
Bunge	0·7	Minimum
„	1·0	Maximum
	Average 0·7	

Milk.

Bunge (1875)	0·24	
„ (1904)	0·30	
Abderhalden	0·23	
Stockman	0·33	Average of 3 estimations
Sherman	0·24	„ 5 „
	Average 0·27	

Thus there is fairly general agreement about the iron content of bread and milk. Taking the averages given above this would make in the bread and milk diet 0·35 mgm. iron per 100 g. rat per day if the whole of the bread and milk was eaten (which was not the case).

There is no such convincing similarity between the figures given for the iron content of green vegetables by different observers.

Green vegetables.

Material	Observer	Iron per 100 g. mgm.
Spinach	Boussingault[1]	4·5
„	Sherman	2·8[2]
„	Bunge	3·2[2]
„	Baldoni	2·0
Cabbage	Boussingault[1]	3·9
„ (outer green) ...	Hausermann	1·4
„ (inner yellow) ...	„	0·4
„ ("edible portion")	Sherman	0·9
Lettuce[3]	„	0·3

[1] This observer's figures are regarded by Stockman as too high.

[2] Corrected as advised by Sherman "Iron in Food."

[3] Presumably the edible portion, *i.e.* the heart.

It would appear unsound to calculate from any average of these data the iron content of the mixed green stuffs given, but it is certain that the diet with green stuffs contains additional iron and it is probable that the iron is in some different form.

2. *Differences in vitamin content.* Bread and milk is usually recognised as containing a bare sufficiency of vitamins but green stuff contains a liberal supply of them.

3. *Differences in other substances.* Other mineral salts, organic groupings such as might be contained in chlorophyll, etc.

From a few days after weaning the experimental animals were fed on the basal diet alone and shortly after puberty, *i.e.* at the age of 90 days, they were subjected to a series of eight bleedings, each of about 1% of the body weight, at intervals of a week. The question was whether these animals would develop a more or less chronic anaemia characterised by a low colour index.

The controls were: (*a*) animals fed on the basal diet plus green stuff, these animals being subjected to an identical series of bleedings; (*b*) animals fed on the basal diet alone and not subjected to bleeding.

In consequence of preliminary experiments it was found necessary to start differential feeding at a very early age because indefinite results were obtained if the rats were fed on a mixed diet (with green stuff) till puberty and differentiation in the diet was delayed till then. The rats which were started on bread and milk only after puberty required a considerable number of these small bleedings (12 or more) before they showed any fall of colour index.

Preliminary Discussion.

At this early stage attention may be called to two papers which appeared while this work was in progress. It will be noted that the blood drawn on each occasion in this work was equal to 1% of the body weight; Geiling and Green [1921] showed that after a haemorrhage of double this size regeneration was complete in 7–10 days. It was therefore reasonable to expect that in this experiment between haemorrhages regeneration would take place to a considerable extent if not completely. The same authors, using purified diets,

showed that a diet deficient in protein, vitamin or mineral matter appreciably delayed blood regeneration after two haemorrhages on successive days.

Jencks [1922] showed that protein causes more rapid regeneration than fat or carbohydrate; vitamin-rich food is specially valuable for blood regeneration.

The work which is detailed below bears out these results but also an intrinsic value may be claimed for it inasmuch as the problem is approached from a slightly different point of view; these observers noted the rate of recovery from an acute experimental anaemia on different diets; this work demonstrates the complementary proposition that a series of haemorrhages insufficient to cause an anaemia (as judged by the lowering of the colour index) on the rich diet is sufficient to cause a moderately persistent anaemia on the poor diet provided growing animals have been kept long enough on it to reduce their reserve stores of blood forming materials to a minimum.

Details of experimental procedure.

Method of bleeding. As fatty changes had been observed in the liver and kidneys in the experiments on cats it was thought wise to avoid anaesthetics, which themselves cause these changes. If the animals were bled from the tail it was a little difficult to control the amount abstracted and resort was made to the use of leeches. The rat was clipped with artery forceps in a piece of sacking; an area on the flank was shorn and a snick was made in the skin with a pair of scissors. To this the leech was applied. This proved an admirable way of extracting blood—for by this means a large number of profuse bleedings can be made on the same rat without trouble. A little experience enabled one to judge approximately from the degree of swelling of the leech how much blood had been abstracted and the exact quantity was determined by the difference in its weight before application and after removal.

Samples of blood, which flowed freely from the incision until stopped by pressure, were taken and examined by the haemocytometer and haemoglobinometer, the apparatus used being those of Bürker and Gowers respectively.

Attention was paid to the following points in the use of the haemocytometer.

1. A fair sample of blood was taken. The blood was allowed to flow freely and the haemocytometer and haemoglobinometer blood was taken so far as possible from the same drop.

2. Pipettes and counting chamber were standardised.

3. The cover glass and Bürker slide were polished till they adhered over parts of the areas in contact, Newton's rings being obliterated there.

4. Solution of corpuscles in the counting fluid (which occurs in Toison and sodium citrate solutions in the case of anaemic bloods) was avoided by using instead mammal Ringer to every 100 cc. of which 3 cc. formalin were freshly added.

5. The mixed fluid was run quickly and evenly through the Bürker chamber. Delay leads to the count being high, as also does any great overflow into the trough.

6. At first 256 squares in each Bürker chamber were counted, but later it was found sufficient to count two sets of 64 squares each in each chamber.

As regards the haemoglobinometer, Gower's standard was finally selected in preference to Haldane's as it was found that its correction constant when tested against a blood of known oxygen capacity remained the same, being 100/83·3 at the beginning of the experiments and 100/83·5 at the end. In the same time the correction constant for the Haldane standard fell from 100/86·5 to 100/92·3, indicating that the standard had faded.

The colour index is expressed in terms of the human standard, this being taken as 1. It is obtained by dividing the percentage of haemoglobin by twice the number of red blood corpuscles.

Tables and a chart showing the results of the experiment follow.

Table I. *Showing the blood condition of normal adult rats on mixed diet.*

Haemoglobin %	Red blood corpuscles in millions per mm.^{3}	Colour index (human index = 1)
81·5	7·34	·56
103	8·74	·59
96	8·57	·56
117	10·6	·55
100	9·03	·55
103	9·27	·56
103	9·27	·56
93	8·40	·55
114	9·63	·59
105	9·53	·55
99	8·90	·56
72	6·50	·55

Average colour index = ·56.

Table II. *Showing the blood condition of control rats put on a diet of bread and milk after weaning and not subjected to bleedings.*

Age in weeks	Haemoglobin %	R.B.C. in millions per mm.^{3}	Colour index (human = 1)	Average C.I.
14	100	9·06	·55	
	112	10·08	·56	
	102	8·32	·61	·56
	100	8·85	·56	
	100	9·16	·54	
	105	9·56	·55	
18	95	8·70	·55	
	107	9·26	·58	·54
	95	8·90	·53	
	90	8·75	·51	
22	93	8·70	·53	
	100	9·48	·53	·53
	102	8·78	·58	
	107	10·76	·50	
26	95	9·25	·51	
	98	9·80	·50	·51
	102	9·80	·52	

Table III. *Showing blood condition of rats put on a diet of bread and milk at weaning and at the age of* 13 *weeks subjected to a series of* 8 *weekly haemorrhages.*

Rat 1.

Age in weeks	Bled cc.	Haemoglobin %	R.B.C. in millions per mm.³	Colour index (human = 1)
14	1·2	90	7·63	·59
15	1·2	76	5·91	·64
16	1·0	67	6·42	·52
17	1·4	54	5·92	·46
18	1·3	39	4·75	·41
19	1·1	51	7·80	·33
20	1·6	51	7·35	·35
21	1·3	—	—	—
22	Not bled	51	7·30	·35
23	,,	56	8·07	·35
24	,,	—	—	—
25	,,	88	11·55	·38
26	,,	88	9·68	·46
27	,,	102	9·65	·53

Average weight during period of bleeding 116 g.
Average amount of blood taken 1·3 cc. = 1·12 % of body weight.

Rat 2.

14	1·4	93	8·16	·57
15	1·5	78	6·26	·62
16	1·3	88	8·04	·55
17	1·8	54	4·86	·56
18	1·1	49	5·98	·41
19	1·3	44	5·24	·42
20	1·3	26	4·22	·31
21	1·4	42	5·56	·37
22	Not bled	54	8·03	·34
23	,,	59	7·48	·40
24	,,	—	—	—
25	,,	83	10·64	·39
26	,,	90	10·36	·44
27	,,	100	9·85	·51

Average weight during period of bleeding 129 g.
Average amount of blood taken 1·4 cc. = 1·08 % of body weight.

Rat 3.

14	1·3	85	7·00	·60
15	1·2	78	5·84	·67
16	1·5	93	7·92	·59
17	1·3	58	5·53	·53
18	1·0	44	5·40	·41
19	·9	41·5	5·28	·39
20	1·7	49	6·46	·38
21	1·3	44	5·65	·39
22	Not bled	49	6·62	·37
23	,,	54	7·54	·36
24	,,	—	—	—
25	,,	99	10·62	·46
26	,,	—	—	—
27	,,	100	9·07	·55

Average weight during period of bleeding 137 g.
Average amount of blood taken 1·3 cc. = 1 % of body weight.

Rat 4.

Age in weeks	Bled cc.	Haemoglobin %	R.B.C. in millions per mm.^{3}	Colour index (human = 1)
14	1·1	98	8·40	·58
15	1·5	—	—	—
16	1·5	83	6·05	·68
17	1·3	68	6·40	·56
18	1·2	73	6·27	·58
19	1·7	54	5·00	·54
20	1·5	49	5·64	·43
21	1·8	59	6·55	·43
22	Not bled	60	8·12	·37
23	,,	63·5	9·82	·32
24	,,	—	—	—
25	,,	93	11·56	·41
26	,,	—	—	—
27	,,	98	9·38	·52

Average weight during period of bleeding 144 g.
Average amount of blood taken 1·5 cc. = 1·04 % of body weight.

Rat 5.

14	1·0	98	8·35	·59
15	1·1	—	—	—
16	1·0	—	—	—
17	1·2	73	6·63	·55
18	1·5	49	5·00	·49
19	1·3	61	6·10	·50
20	1·4	66	8·45	·39
21	1·4	46	6·15	·37
22	Not bled	41·5	5·94	·35
23	,,	44	7·35	·30
24	,,	—	—	—
25	,,	100	10·70	·47
26	,,	—	—	—
27	,,	100	9·55	·52

Average weight during period of bleeding 112 g.
Average amount of blood taken 1·3 cc. = 1·16 % of body weight.

Table IV. *Showing the blood condition of control rats fed on bread and milk and green stuffs and at the age of* 13 *weeks subjected to a series of* 8 *weekly haemorrhages.*

Rat A.

Age in weeks	Bled cc.	Haemoglobin %	R.B.C. in millions per mm.^{3}	Colour index (human = 1)
14	1·0	98	8·46	·58
15	1·1	107	8·52	·63
16	1·1	84	7·36	·57
17	1·3	88·5	8·20	·54
18	1·2	84	7·62	·55
19	1·6	84	7·78	·54
20	1·5	94	8·40	·56
21	1·9	98	8·92	·55
22	1·9	90	8·06	·56

Average weight during period of bleeding 143 g.
Average amount of blood taken 1·4 cc. = 1 % of body weight.

Rat B.

Age in weeks	Bled cc.	Haemoglobin %	R.B.C. in millions per mm.³	Colour index (human = 1)
14	1·1	110	9·33	·59
15	1·1	91	7·35	·62
16	1·3	98	8·42	·58
17	1·2	86	7·56	·57
18	1·1	89	8·26	·54
19	1·3	95	8·66	·55
20	1·9	93	8·20	·57
21	1·9	91	8·22	·55
22	2·0	91	8·32	·55

Average weight during period of bleeding 140 g.
Average amount of blood taken 1·4 cc. = 1 % of body weight.

Rat C.

14	·9	108	9·33	·58
15	1·3	93·5	9·16	·51
16	1·4	84	8·42	·50
17	1·4	103	9·38	·55
18	1·2	98	9·06	·54
19	1·3	95	8·80	·54
20	1·8	93	8·48	·55
21	1·7	98	9·07	·54
22	1·5	98	9·00	·54

Average weight during period of bleeding 140 g.
Average amount of blood taken 1·4 cc. = 1 % of body weight.

Rat D.

14	·8	105	8·74	·60
15	·9	103	7·98	·65
16	1·4	98	8·40	·58
17	1·5	95	7·68	·55
18	1·2	98	8·64	·57
19	1·4	93	8·76	·53
20	1·7	88	7·96	·55
21	1·9	88	8·10	·54
22	1·3	86	8·08	·53

Average weight during period of bleeding 139 g.
Average amount of blood taken 1·3 cc. = 1 % of body weight.

It is to be noted that the first bleeding was followed by a rise in the colour index instead of by a fall. This increase in colour index has been specially noticed by Hough and Waddell [1916].

Conclusion.

Rats fed from the time of weaning on white bread and whole milk and subjected to a series of eight bleedings each amounting to 1 % of the body weight show a fall in colour index which persists for 2–3 weeks after the bleeding is stopped; whereas controls fed from the time of weaning on this diet plus green stuff show a comparatively inappreciable fall of colour index. Controls fed on this diet but not bled also show a comparatively inappreciable fall of colour index.

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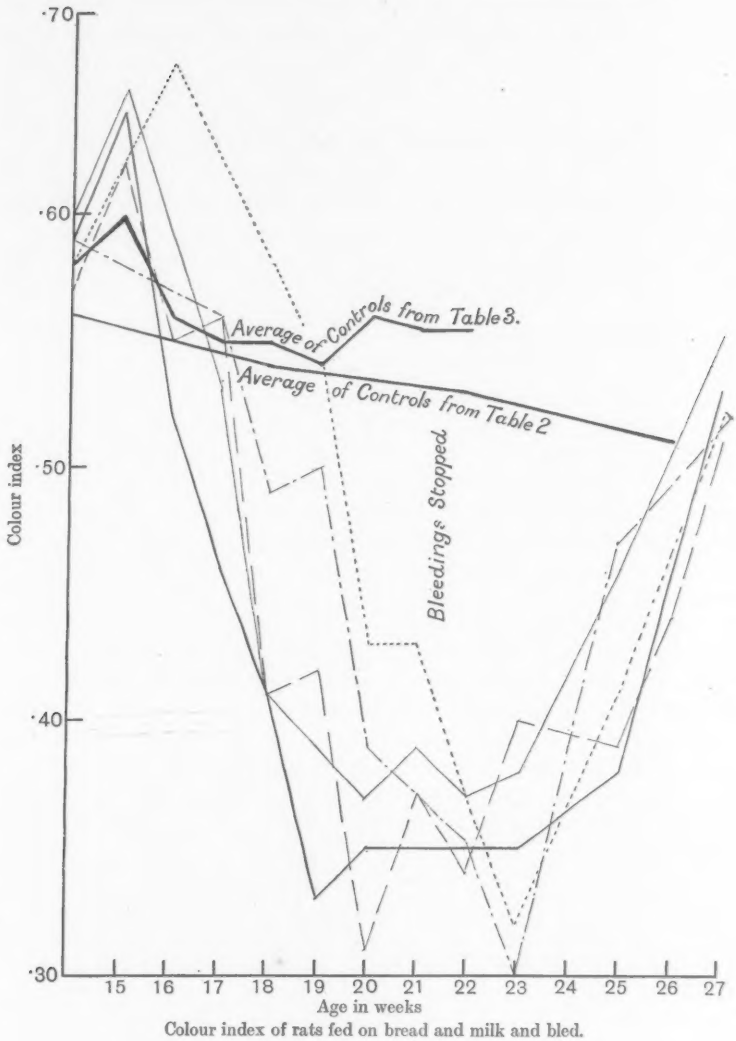

Colour index of rats fed on bread and milk and bled.

REFERENCES.

Geiling and Green (1921). *Proc. Soc. Exp. Biol. Med.* **18**, 191.
Hough and Waddell (1916). *Amer. J. Physiol. Proc.* **40**, 136
Jencks (1922). *Amer. J. Physiol.* **59**, 240.
Sherman (1907). Iron in Food. *U.S. Dept. of Agriculture Office of Experiment Stations Bulletin*, 185.

XXI. STUDIES IN ANAEMIA. II.

By JAMES MATTHEWS DUNCAN SCOTT (*John Lucas Walker Student*).

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(*Received January 22nd, 1923.*)

Comparison of methods of producing an anaemia characterised by a low colour index.

Most investigators of anaemia, in making observations on such points as basal metabolism, circulation rate and blood reaction, and also on such questions as the influence of iron, etc., on blood regeneration, have confined themselves to acute experimental anaemia produced by bleeding. Such an anaemia is at best a transitory phenomenon, the blood soon being regenerated either from a reserve of blood forming materials or from constituents of the diet. The production of a sub-acute anaemia by bleeding and feeding as described in Part I of this paper [1923] is a process so tedious and laborious that before proceeding further it seemed desirable to search for some easier method of producing a more permanent anaemia, and the expedient of breeding from females which had been kept on a diet poor in iron (bread and milk only) from the age of puberty till ten months was adopted. These rats showed practically no anaemia themselves, their colour index being about 0·52 as against the normal 0·56. Healthy males were used for breeding and the process was looked upon as a case where deficiency of some chemical substance in the mother caused a deficiency of the same substance in more marked degree in her offspring.

This was the course adopted by M. B. Schmidt in 1912. Schmidt [1912] found that if a diet of rice and milk were continued in growing mice they gradually became anaemic and were stunted in their growth. The offspring of such mice were studied through several generations throughout which the feeding was continuously "iron-free." If now iron were given to one of a litter of such stunted anaemic mice it quickly outgrew the others and acquired a high percentage of haemoglobin and a nearly normal blood count.

Attention may also be drawn to the work of Brinckmann [1921], who showed that after feeding young guinea-pigs on iron-poor food anaemia of chlorotic type ensues.

In view of our more intimate knowledge of the rat and his greater range of usefulness in experimental procedure it seemed desirable to repeat the experiments with these animals and to institute a comparison between the

ease of this process and the process adopted in Part I of this work for the production of anaemia and to compare the degree of chronicity of the anaemias produced.

Rats were bred from healthy fathers and from mothers which had been kept till ten months old on white bread and whole milk. The results of blood examination of the offspring at various ages are shown in Tables I, II and III. It will be seen that as compared with normal controls and with control rats from the same litters which had green stuff added to their diet they show when young a marked depression of the colour index of the corpuscle and that this shows a tendency to ameliorate naturally as the animals get older. Males recover faster than females.

Table I. *Showing blood condition in normal rats at ages* 40 *and* 60 *days.* For adult rats see Table I, Part I.

Sex	Hb %	R.B.C. in millions per mm.³	Colour index (human = 1)	Average colour index
Age: 40 days. Average weight: 92 g.				
M.	85	7·70	·55	
M.	78	6·33	·62	·57
M.	80·5	7·36	·55	
F.	85·5	7·87	·54	
F.	88	8·00	·55	·56
F.	93	8·10	·58	
Age: 60 days. Average weight: 104 g.				
M.	95	8·86	·54	
M.	85	7·54	·57	·57
M.	93	7·80	·60	
F.	95	8·74	·54	
F.	94	7·73	·61	·57
F.	89	7·67	·58	

Results.

Young rats bred from mothers which had been kept on a diet of white bread and whole milk from puberty till ten months old showed a marked fall in the colour index of the corpuscle owing to the fact that the haemoglobin content of the blood had fallen while the number of corpuscles had risen. This is a phenomenon to which attention may be called. The anaemia was fairly chronic but tended to ameliorate naturally in time on a diet of white bread and whole milk. Its experimental production was easy compared with the method previously adopted in Part I. Control rats from the same litters fed on the same diet but with green stuff added showed a speedy recovery from the anaemic condition.

Discussion.

Hartwell [1921, 1922] has reported work in which she shows that a diet of white bread and milk is adequate for nursing mother rats and indeed gives the best growth curves for sucklings. Sherman and Muhlfeld [1922] also regard a very similar diet of whole milk powder and ground whole wheat as

Table II. *Showing blood condition at various ages of rats bred from mothers ten months old, mothers and young having been kept on a diet of bread and milk.*

A. Females.

Age in weeks	Hb %	R.B.C. in millions per mm.³	Colour index (human = 1)	Average colour index
8	68·5	10·60	·33	
	68·5	10·22	·34	·33
	68·5	10·58	·33	
10	73	10·78	·34	
	73	11·82	·31	·33
	73	11·00	·33	
12	78	12·00	·33	
	78	11·42	·34	·34
	85	12·22	·35	
16	93	11·18	·42	
	88	10·70	·41	·42
	81	9·72	·42	
22	100	10·32	·48	
	105	10·40	·50	·50
	101	9·67	·52	
24	100	11·20	·45	
	117	11·02	·53	
	112	10·40	·54	
	107	10·52	·51	·51
	100	9·56	·52	
	110	10·60	·51	

B. Males.

8	76	11·36	·34	
	61	9·60	·32	·35
	63	8·10	·39	
10	73	11·10	·33	
	73	11·20	·33	·32
	73	11·90	·31	
13	102	12·58	·41	
	61	9·14	·33	·35
	78	12·88	·30	
18	95	10·48	·45	
	95	10·05	·47	·46
	98	10·63	·46	
22	110	9·92	·55	
	105	9·58	·55	·54
	102	9·76	·52	

Table III. *Showing blood condition at various ages of control rats from the same litters which had been fed since weaning at four weeks old on green stuff in addition to bread and milk.*

Age in weeks	Hb %	R.B.C. in millions per mm.³	Colour index (human = 1)	Average colour index
8	100	10·60	·47	
	95	9·30	·51	
	100	8·82	·56	·49
	96	10·34	·46	
	94	9·86	·48	
	91	9·56	·47	
10	104	10·10	·51	
	104	11·46	·46	·48
	107	11·60	·46	
13	102	9·42	·54	
	93	8·30	·56	·54
	100	9·65	·52	

adequate for the growth, reproduction, and successful suckling of the second generation. While these conclusions might be justified from the evidence of growth curves alone, yet the work reported in this paper shows that this cannot be interpreted as meaning that these diets are adequate for blood formation. While it is true that we do not know what the blood condition of Miss Hartwell's rats was like, and it may or may not have been abnormal even though growth was normal, the obvious difference between her rats and these was that her rats were bred of robust mothers, who presumably had given them at birth what these rats lacked and were unable to acquire from the poor diet on which they were fed. It would appear that growth curves alone over a limited period of time are not a sufficient test of the adequacy of a diet. An animal may exhibit a satisfactory growth curve and yet be flabby. Further it may have latent a diathesis which manifests itself as anaemia in its offspring.

Conclusion.

A simple method of producing a comparatively chronic anaemia characterised by a low colour index and by a tendency to natural cure is described.

REFERENCES.

Brinckmann (1921). *Z. Kinderheilk*, **30**, 158–94.
Hartwell (1921). *Biochem. J.* **15**, 149.
—— (1922). *Lancet*, i. 323.
Schmidt (1912). *Verh. deutsch. path. Gesellsch.* **15**, 91.
Scott (1923). *Biochem. J.* **17**, 157.
Sherman and Muhlfeld (1922). *Proc. Soc. Exp. Biol. Med.* **19**, 76.

XXII. RESPIRATORY EXCHANGE IN FRESH-WATER FISH.

PART VI. ON PIKE (*ESOX LUCIUS*)

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(*Received February 3rd, 1923.*)

Pike form a small family of soft-rayed fishes pretty generally distributed over the rivers and lakes of Europe, Northern Asia and North America. In the old world one species only—*Esox lucius*—is common. Its eastward range is not known, as far as we are aware, but it extends into Lapland in the north and into central Italy in the south; it appears however to be absent from the Iberian Peninsula. It has an elongate rather compressed body covered with small scales, a long head with long and spatulate snout, and a very large mouth armed with strong teeth in the jaws and bands of smaller teeth in the palate and tongue. The teeth point backwards or can be depressed so as to offer no obstruction to any object entering the gape, but prevent its withdrawal in the opposite direction. The pike is the most voracious of fresh water fishes, and many writers have compared it to the shark with regard to the quantity of food it consumes. It leads rather a sedentary than a roving life, and prefers lakes and the sluggish reaches of rivers to strong currents or agitated waters. The dorsal and anal fins are placed far back in the tail, thus greatly increasing the propelling power of the fish, and though relatively poor swimmers pike are excelled by no other fresh-water fish in rapidity of motion when by a single stroke of the tail, they dart upon their prey or out of reach of danger. They appear to be very hardy and we found they could stand confinement remarkably well.

On account of their sluggish disposition and yet great voracity it seemed of interest to determine their oxygen requirements. The fish used for experiment were about 12 inches in length and varied in weight from 110 to 160 g. When placed in the tank they remained very sluggish during the experimental period and though there were plenty of minnows in the tank, the pike did not exhibit their usual voracity, and specimens killed from time to time showed little or no food in the stomach. Probably they were scared by the necessary handling.

The method adopted was that fully described in former papers of this series [1914, 1 and 1922, 1, 2].

EXPERIMENTAL.

At low temperature. Four fish were taken weighing respectively 150, 110, 100 and 140 g. Total weight 500. Initial temp. of water 5·7°, final temp. 6·1°. Duration of experiment 18 hours 57 minutes. 275 cc. commercial oxygen were added during the experiment.

Total free and combined CO_2	at beginning 1513·2 cc.	at end 1673·6 cc.	diff. 160·4 cc.
„ oxygen	„ 5208·1	„ 4963·8	„ 244·3
„ nitrogen	„ 18975·2	„ 18973·6	„ 1·6

Error in nitrogen 0·0084 %.

At medium temperature. Five fish, 125, 105, 145, 110 and 155 g. respectively. Total 640 g. Initial temp. of water 11·2°, final temp. 13·7°. Duration of experiment 4 hours 17 minutes. 275 cc. oxygen added during experiment.

Total free and combined CO_2	initial 2164·5 cc.	final 2297·6 cc.	diff. 133·1 cc.
„ oxygen	„ 4362·5	„ 4181·6	„ 180·9
„ nitrogen	„ 15655·9	„ 15658·9	„ 3·0

Nitrogen error 0·0199 %.

Five fish total weight 595 g. Initial temp. of water 16·6°, final temp. 16·65°. Duration of experiment 4 hours 14 minutes. During the experiment 275 cc. oxygen added.

Total free and combined CO_2	initial 1874·4 cc.	final 2039·9 cc.	diff. 165·5 cc.
„ oxygen	„ 4534·8	„ 4341·2	„ 193·4
„ nitrogen	„ 16267·1	„ 16275·0	„ 7·9

Nitrogen error 0·048 %.

At high temperature. Five fish, 145, 155, 135, 160 and 110 g. respectively, total 705 g. Initial temp. of water 22·9°, final 22·2°. Duration of experiment 4 hours 8 minutes. 275 cc. oxygen added.

Total free and combined CO_2	initial 1781·2 cc.	final 2026·4 cc.	diff. 245·2 cc.
„ oxygen	„ 4514·3	„ 4215·4	„ 298·9
„ nitrogen	„ 16419·8	„ 16398·0	„ 21·8

Nitrogen error 0·13 %.

These results are gathered together in the following table.

Table I.

Temperature °C.	Oxygen consumed per fish per hour cc.	Oxygen consumed per kilo of fish per hour cc.	CO_2 evolved per fish per hour cc.	CO_2 evolved per kilo of fish per hour cc.	Respiratory quotient
5·7– 6·1	3·06	24·44	2·01	16·08	0·66
11·2–13·7	8·45	65·99	6·22	48·56	0·74
16·6–16·65	9·14	76·79	7·82	65·71	0·86
22·9–22·2	14·46	102·58	11·87	84·18	0·82

In this case again the oxygen consumed per fish is approximately proportional to the rise in temperature, and if the oxygen values are plotted against temperature the curve is nearly a straight line.

The respiratory quotients at all temperatures were approximately normal for carnivorous animals.

Table II. *Oxygen consumption per kilo of fish.*

Temperature °C.	Pike	Gold fish	Eels
5°–6°	24·44	16·07	9·28
about 16°	76·79	50·09	44·40
22°	102·58	83·81	61·29

On comparing the oxygen consumption of pike with that of eels and gold fish, it will be seen from Table II that at the various temperatures pike require more oxygen than gold fish in the ratio of about 3 : 2, and that gold fish in this respect occupy a position midway between pike and eels.

Quantity of oxygen in the water at the asphyxial point for pike.

It seemed of interest to determine the behaviour of pike under reduced oxygen tension and measure the quantity of oxygen in the water at the asphyxial point.

Experiments were carried out at a medium temperature and the apparatus and method fully described in Part II of this series of papers [1914, 2] were used. No experiments were made at low temperatures as it did not seem likely that it would be possible to reduce the oxygen in the water to the asphyxial tension within a reasonable time, and the fish at our disposal were too large for the small apparatus used for gold fish in Part IV [1922, 2].

A constant stream of nitrogen was passed through the experimental bottle during the night to reduce the oxygen tension in the water and the fish were introduced the following morning.

Four fish weighing respectively (1) 90, (2) 150, (3) 152 and (4) 120 g. were selected. Two fish were placed in the bottle at 10·58 a.m., the temperature of the water being 15·3°. Both fish remained inactive at first and the respiration rate was about 77 per minute. Minute bubbles of gas were seen to rise occasionally from the smaller fish, but no sign of distress was apparent in either and movement was only occasional. At 11.40 a.m. the other two fish (3 and 4) were introduced, and nitrogen was circulated through the water by the pumping apparatus. The bubbling of fresh nitrogen through the water sampling tube and the pumping through the spraying tube were carried on simultaneously.

At 12.45 p.m. one of the fish began to show marked activity and was seen to rise to the surface. All the fish then became more active and rose to the surface frequently. At 1.20 p.m. one of the fish gave a violent leap out of the water, which was repeated a little afterwards. The frequency of respiration of one of the fish at 1.45 p.m. was 60–70 per minute. Increased activity, accompanied by vigorous leaps out of the water, was observed until about 2.45 p.m., after which the fish again became sluggish and their movements more spasmodic with longer intervals of rest. At 3.45 p.m. one fish gave a violent leap and then turned over. After some spasmodic movements the fish settled on its back, with occasional convulsive gasps. The other fish showed signs of getting into the same condition. At this point a sample of water was withdrawn for analysis, and the current of nitrogen then replaced by a rapid current of oxygen. At 4.45 p.m. the fish which had been most affected again assumed a normal position. On removal to the outdoor tank the fish

appeared to have quite recovered and continued perfectly healthy for many weeks, and were ultimately killed for other purposes.

Analysis of sample of water, the temperature of which when collected was 14·1°.

The gas dissolved in the water sample (45·995 cc. of water) was pumped out in the usual way. It exerted a pressure of 10·225 mm. Hg at 11·64° and constant volume 36·62 cc.; after shaking with 40 % KOH it exerted a pressure of 1·83 mm. Hg at 12·08°; and after shaking with alkaline pyrogallol, a pressure of 1·80 mm. Hg at 12·60°. The volumes of CO_2 (free and combined), oxygen, and nitrogen dissolved per litre of water were therefore 84·38 cc., 0·334 cc., and 18·025 cc. respectively, measured at S.T.P. The coefficient of absorption of O_2 in water at 14·1° is 0·03479.

Hence the tension of dissolved oxygen was $\frac{0{\cdot}334}{10 \times 0{\cdot}03479} = 0{\cdot}96$ % of an atmosphere, *i.e.* 7·30 mm. Hg.

Evidently pike, like gold fish and eels, can exist at tensions considerably below normal without harm.

Limits of temperature within which pike can live.

In order to determine the highest temperature at which pike can live, a fish *A* weighing 150 g. and another *B* weighing 107 g. were placed in a tub of cold water at 11°, and the temperature was very gradually raised to 18–19° by careful addition of warm water. The water was kept fully oxygenated by spraying oxygen through by means of a circular lead coil perforated with fine holes. The temperature was very gradually raised, but no sign of discomforture was observed until the temperature had reached 27°. The rates of respiration noted at various temperatures were as follows:

Temperature	11°	18°	22°	26°	27°	29°
Respiration rate	56	47	45	40	55	63

At 27° the respirations were markedly deeper and the fish became more active. Movements became slightly convulsive in the neighbourhood of 30°, and at this temperature both fish turned over. The fish were then immediately removed to cold water, and on return to the outside tank quickly revived and appeared to have suffered no harm. They were alive and healthy many weeks after the experiment.

In our experience pike are remarkably hardy in confinement, and consequently very suitable for experiments such as have been described, and, as we hope to show later, for experiments on the effects of toxic substances.

We take this opportunity of expressing our thanks to Mr A. R. Peart of the Berkshire Trout Farm, Hungerford, for his kindness in procuring suitable fish and for much most valuable advice and information.

REFERENCES.

Gardner and Leatham (1914, 1). *Biochem. J.* **8**, 374.
—— —— (1914, 2). *Biochem. J.* **8**, 591.
Gardner, King and Powers (1922, 1). *Biochem. J.* **16**, 524.
Gardner and King (1922, 2). *Biochem. J.* **16**, 729.

XXIII. THE RATE OF REPRODUCTION IN ARTIFICIAL CULTURE OF *COLPIDIUM COLPODA*.

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(*Received February 6th, 1923.*)

Introduction.

That protozoa are important factors in soil economy is becoming increasingly evident since there is no question that large numbers of them pass through the various phases of their life history in the soil, and during their life influence the other members of the population. In a previous paper [Cutler, Crump and Sandon, 1922] it has been shown that the numbers of active protozoa of various species change rapidly from day to day without any obvious reference to gross environmental changes. Thus in the course of a year's daily counts in field soil, no correlation could be traced between the fluctuations in numbers of six species of protozoa and the rainfall, temperature or moisture content, and moreover the species appeared in the main to be living independently of one another.

It therefore appears that these variations in numbers are the expression of the animal's life cycle, or else that there is some obscure factor or combination of factors at work in the soil limiting activity. This second alternative is of course possible. If, however, the first suggestion is the true one growth in pure cultures should follow the same general course as obtains in the soil. For the two species tested, *Colpidium colpoda*, a ciliate, and *Oicomonas termo* (Ehren.), a flagellate, this is the case; for after the first few days of incubation, during which the culture is becoming populated, irregular variations in numbers occur, which are in every way comparable to the soil fluctuations.

The experiments detailed below were undertaken with a view to elucidating these changes, and to finding what are the factors in liquid culture determining the variations in reproductive rate and death.

From the point of view of soil biology such information is essential, but it is of added importance in respect of the various theories that have been advanced in explanation of growth. This has been dealt with extensively for the metazoa, metaphyta, and bacteria, but little is known regarding protozoa. Recently, however, Robertson [1921] has published experiments which he believes demonstrate that in certain ciliates growth is auto-catalytic

in character similar to that which has been recorded for higher organisms. Further research is necessary either to prove or disprove this view but the evidence adduced is not convincing.

The following account deals only with the earlier stages of growth in mass culture, later stages having been dealt with in detail by various authors.

In a future communication it is hoped to describe experiments on single cells isolated into drops of culture media of varying volumes.

Methods.

The first essential in work of this kind is a rigorous standardisation of the cultural methods, so that any experiment can be repeated, or carried out in duplicate, with a reasonable expectation of obtaining consistent results, the organisms themselves being as far as possible the only variable factors.

Two organisms, whose source will be considered later, have been used throughout these experiments, *Oicomonas termo* and *Colpidium colpoda*. The method of estimating growth has been to count the number of animals in representative samples of the culture fluid in a counting chamber. The most convenient type for counting organisms of this size is the Cropper ruling, where an area of 2·5 sq. mm. is divided up into 625 squares of 0·04 sq. mm. area, the whole chamber being 0·1 mm. deep. The experimental error of the counts depends upon the number of animals counted, provided that the organisms are uniformly suspended in the fluid; then, if x is the number counted, the actual number present will lie between the limits $x \pm \sqrt{x}$ [Student, 1907]. In every case the number per cc. is given[1]. The animals are killed before counting by adding a small drop of lugol to the counting chamber. The experiments have all been carried out in the following way: 100 cc. flasks of quartz or Jena glass containing a known amount of medium (10 or 20 cc.) are used for the cultures. The number of organisms in the parent culture is counted and a quantity of liquid varying from 0·1 to 1·0 cc., according to the strength of inoculum required, is pipetted over into a new flask. In every case where the numbers of organisms in the sub-cultures have been counted immediately after inoculation the expected number has been found. The cultures are incubated at 19°.

Preliminary work suggested the following experimental conditions, variation in which would introduce errors:

1. Composition of medium.
2. Glass ware.
3. Aeration.
4. Temperature of incubation.
5. Strain of organism.
6. Food supply.

[1] The reproductive rate for any time is calculated from the formula: $\frac{\log B - \log A}{\log 2}$ where A = no. at beginning of time and B at the end.

1. *Composition of medium.* For comparative work organic infusions are wholly unsuitable, as it is impossible to reproduce them exactly. For this reason hay infusion has been discarded, although both *Oicomonas* and *Colpidium* thrive in it, and a synthetic medium has been used instead. In the first experiments on *Colpidium* Peters's "ammonium glycerophosphate medium" was used [Peters, 1921], or else one in which 0·06 % ammonium phosphate and 0·25 % saccharose were substituted for the glycerophosphate; the later experiments were carried out in a variation of Peters's glucose and lactate medium which contains the following compounds:

Na_2HPO_4	0·001 %	$MgSO_4, 7H_2O$ ...	0·0001 %
Ammonium lactate	0·01	$CaCl_2$	0·002
KCl	0·03	Glucose	0·04
NH_4Cl	0·03		

Colpidium has flourished on this medium for more than five months. According to Peters the optimum growth is obtained in this ciliate when the reaction of the medium is initially p_H 7·0 to 7·4. The medium has therefore always been adjusted to an initial reaction of approximately p_H 7·2, but satisfactory growth has been obtained at values outside these. Thus cultures starting at p_H 7·6 and 6·8 have shown apparently normal growth, and judging by the fact that Dale [1913] found the limiting values for growth in *Paramoecium caudatum* to be p_H 5·0 and 9·0 the range is probably very much wider.

Also a series of experiments made by S. M. Nasir in this laboratory showed that both ciliates and flagellates could live and reproduce in artificial media of p_H 3·9.

Peters's suggestion of adding phenol red in minute quantities to the cultures has also been followed, so that changes in acidity and alkalinity during the life of the culture can be noted; the routine procedure is to add two drops of the indicator to 20 cc. of medium. The final modification of the medium very gradually becomes alkaline as growth proceeds.

2. *Glass ware.* At an early stage in the experiments it was found that certain flasks, notably those made of Bohemian glass, had a bad effect on the growth of the organisms; in some cases no growth occurred at all. All the recorded experiments have been carried out in Jena glass or quartz flasks which give uniform results and do not affect the p_H value of the medium even after repeated sterilisation in the autoclave.

3. *Aeration.* Differences in aeration were found to have a marked effect on cultures of *Oicomonas*. Three series of cultures, each containing three parallels, were put up in the ordinary way: the first series were plugged with cotton wool, the second with corks through which a piece of bent glass tubing ran, and the third were attached to a filter pump so that a steady stream of air was drawn through them. In the second and third series the tubes leading to the outside air were so bent that no contamination of the flasks could occur. The results of this experiment are shown in Fig. 1. It is obvious that extreme aeration has a very disturbing effect on the growth of the cultures, and that

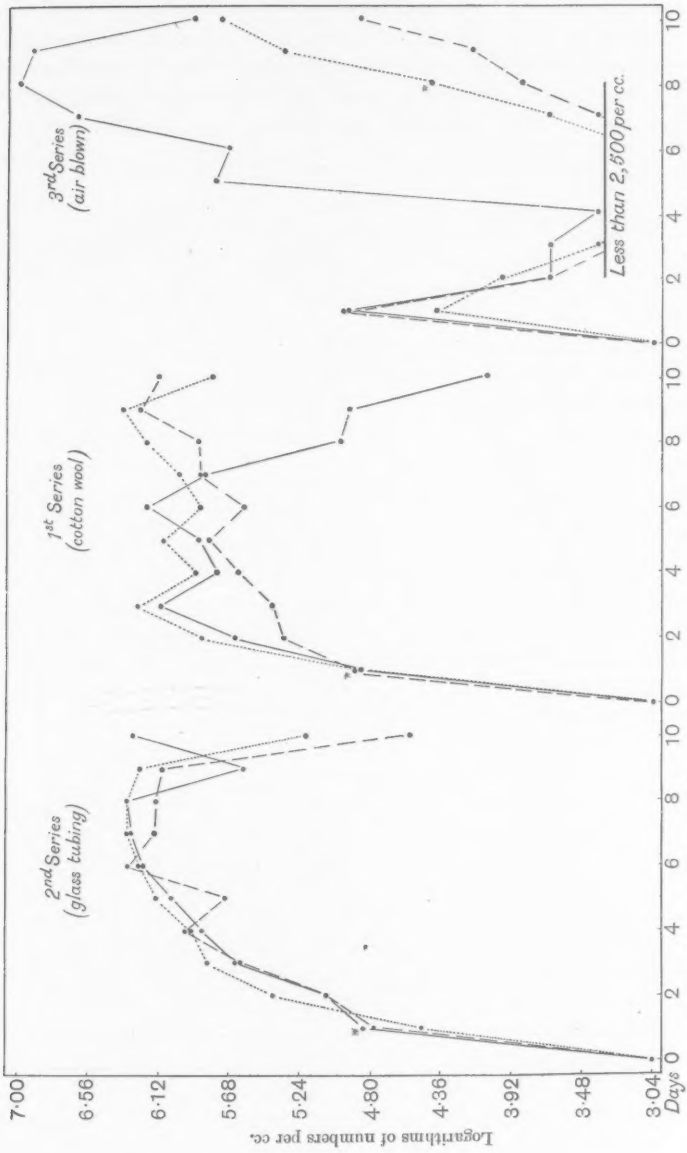

Fig. 1. Curves showing the effect of aeration on the reproduction of *Oicomonas*. The asterisks mark the points where conjugation forms were first observed.

better parallels are obtained when the aeration is absolutely constant (2nd series) than where there is even a slight variation (1st series).

4. *Temperature of incubation.* The incubation temperature throughout all the experiments has been as far as possible 19°; the effect of changing temperature is clearly shown from the results of the following experiments where two sets of flasks were incubated at 19° and 24° respectively (Fig. 2). Although

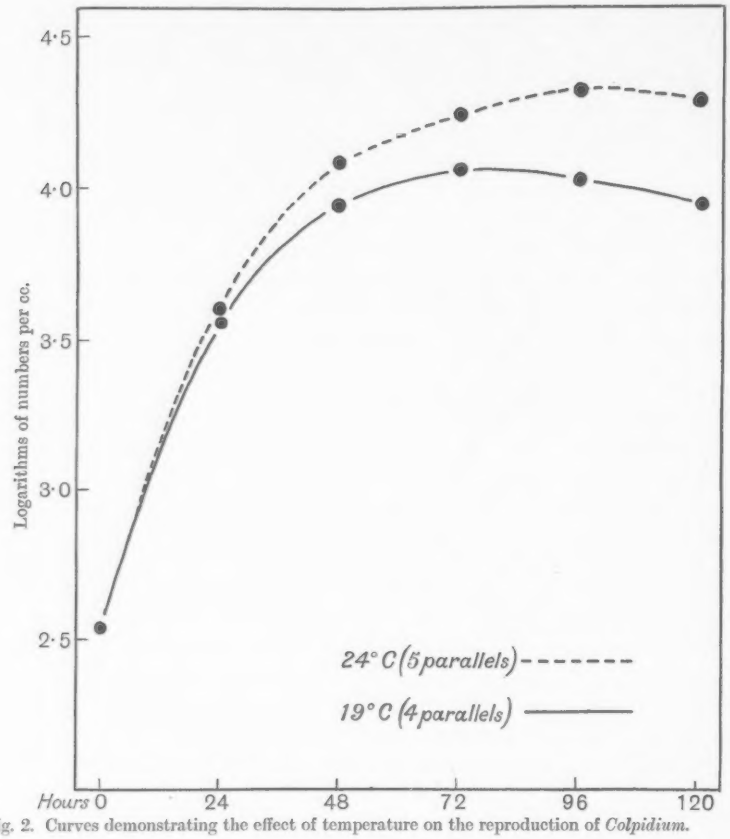

Fig. 2. Curves demonstrating the effect of temperature on the reproduction of *Colpidium*.

24° would appear to encourage more rapid, and even greater, reproduction in *Colpidium*, it is possible to maintain better experimental conditions at the lower temperature, as it approximates more nearly to that of the room and the shock of transferring the flasks from the incubator to the bench is very much less. In the experiment involving three hourly counts (cf. p. 180) a bath kept at 19° was arranged on the bench by the microscope and each flask was placed in it while the counts were made; McKendrick and Pai [1911] found this

a very necessary precaution where working with bacteria at incubator temperatures of 37°; at the lower temperatures it is probably an unnecessary refinement.

5. *Strain of organism.* The variation introduced by the strain of organism is probably considerable, since different strains show consistent differences in many characters. Thus, known differences in strain include such characters as size, rate of multiplication and frequency of conjugation [Jennings, 1920], resistance to heat [Hutchison, 1913; Jollos, 1913], the readiness with which conjugation occurs [Hopkins, 1922] and resistance to poisons [Jollos, 1913]. All the experiments on *Oicomonas* have been carried out on a single strain, the original culture being obtained from soil from Barnfield dunged plot; it is believed that the cultures came from a single cyst, though, owing to the small size of this organism, it is difficult to assert this positively. The cultures of *Oicomonas* are contaminated with at least three species of bacteria. Conjugation is of very frequent occurrence in *Oicomonas termo* thus making it from the point of view of the present work an unsatisfactory species to use. After the first appearance of the large conjugation forms (marked in each figure) the counts are subject to a considerable source of error; up to this point, however, they are accurate within the limits of experimental error. In the case of *Colpidium* all the experiments have been carried out on the same strain. The original stocks were given to us by Dr Peters and each had been derived in the first instance from a single cell. All our stocks of this ciliate are contaminated by a very small, stout, gram positive bacterium, which is present only in small numbers. Conjugation has never been seen in any of the cultures although they have been under close observation for more than six months, and only a very few isolated cysts have been found during that time.

6. *Food supply.* In the growth of a culture two factors are involved: the growth in size of the individuals, and the increase in their numbers, an increase, brought about in the cases under consideration, by binary fission. To attack the problem of growth is therefore by no means simple. The actual increase in size in any one animal depends in great part on the food supply[1], at least in young cultures; for this reason the most profitable method has seemed to be to provide a surplus of acceptable food and then to treat the increase of numbers as indicative of the actual increase in protoplasm. The *Colpidium* cultures have been fed either at inoculation or after 24 hours' growth with a pure culture of *Sarcina lutea* in sufficient quantity to ensure that a supply of it is present throughout the experiment. In the case of *Oicomonas* the contaminating bacteria provide abundant food. Even with these precautions the increase in the amount of living protoplasm in a culture can be found only by regarding both reproductive rate and average size of the animals. At present, however, our attention is confined to rate of reproduction.

[1] A culture of colpidia in which the mean length of 30 animals was 15μ and the mean breadth 5μ and in which there was a scarcity of bacteria was fed with sarcina; on the following day the mean dimensions for 30 animals were 20$\mu \times 11\mu$.

Results.

The general course of a curve plotted from counts made at 12 or 24 hourly intervals on a mass culture of *Colpidium* is roughly sigmoid for a period varying from 2–6 days. There is then a fall in the numbers, and after this first maximum the curve becomes wholly irregular approximating closely to the type of curve obtained by counting protozoa in soil. As conjugation has not been taking place in any of the cultures it is obvious that death is the cause of the drops in the numbers. Experiment has shown, however, that death occurs in the earlier stages of the culture as well, and that the initial smoothly rising part of the curve can be resolved into an irregularly rising line by making counts at shorter intervals of time[1]. For this purpose three hourly counts were made extending over a period of 102 hours on eight cultures, four being made from a 24 hour old parent and four from one of 96 hours. In Figs. 3 and 4 curves derived from a typical culture from each series are shown. In each case the curve obtained from the 12 hourly observations shows a steady rise, while the 3 hourly line rises and falls irregularly. If only the 24 hourly observations are plotted, the curves are again completely changed, as in each case 2 maxima, which occurred at night, escape notice. It is not surprising that such being the case, it has not proved possible to apply the commonly accepted auto-catalytic formula[2] to any of the colpidium curves.

Death following inoculation.

A further complication, which must be recognised, before any general theory of growth and reproduction can be made, lies in the fact that death also occurs under certain conditions immediately after inoculation. Such is the case in cultures made from old parents. This is best illustrated by reference to the following experiments on *Colpidium* and *Oicomonas* (Figs. 5 and 6). In these, sub-cultures were made from the same stock culture at intervals of 12 or 24 hours and the numbers of protozoa in the sub-culture were counted after 12 and 24 hours and afterwards at 24 hourly intervals. During the first 12 hours after inoculation the sub-cultures from the older parents show a decrease in numbers; this, however, would pass unnoticed if the counts at 12 hours were omitted and the curve would then show the lag which is commonly found in bacterial cultures. Thus McKendrick and Pai [1911] with bacteria counted at half-hourly intervals found a lag when their cultures were derived from old parents (14 days as against 1–3 hours), which did not appear in cultures from young parents. Penfold [1914] found that the older the parent culture in the case of *B. coli* within limits, the longer is the lag period in the sub-cultures. In the protozoa Calkins [1919] has found in cultures of *Uroleptus* derived from single ex-conjugants, that old age in the parents leads to a series of low vitality, as evidenced by the duration of life and by reproductive

[1] In the case of certain bacteria Wilson [1922] finds that there is a normal death rate even during the period when the maximum rate of growth is proceeding.

[2] $\log \frac{x}{a-x} = k\,(t - t_1)$.

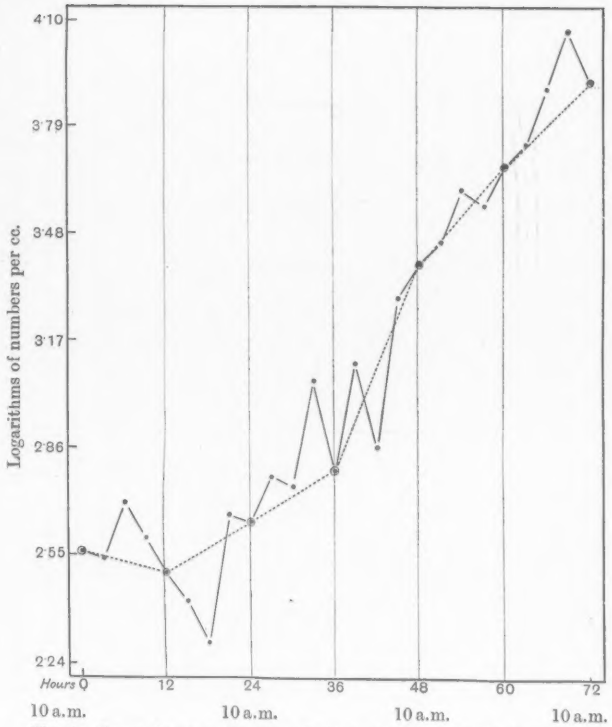

Fig. 3. Curve showing the reproductive rate of *Colpidia* from counts made at three hourly intervals. The broken line gives the curve obtained if 12 hourly counts had been made. The age of the parent was 96 hours.

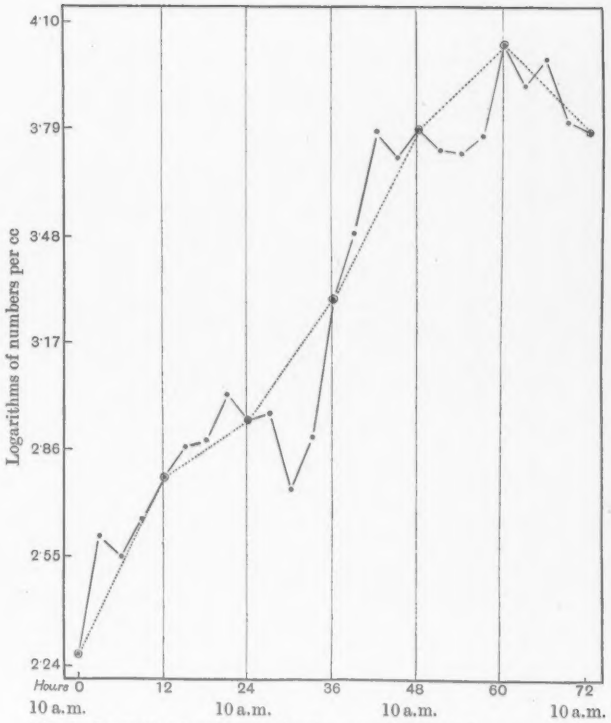

Fig. 4. Curve showing the reproductive rate of *Colpidia* from counts made at three hourly intervals. The broken line gives the curve obtained if 12 hourly counts had been made. The age of the parent was 24 hours.

ability, while young parents produce series of relatively high vitality. Robertson [1921] also adduces a certain amount of evidence bearing on this point. Starting from single individuals of *Enchelys* he finds that on an average

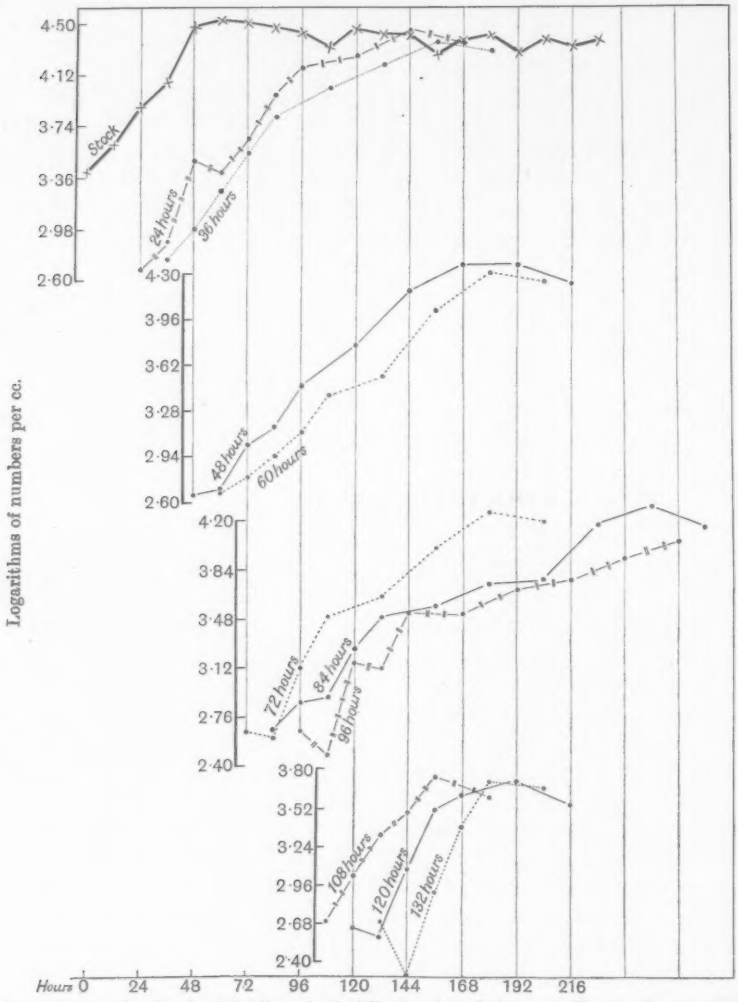

Fig. 5. Curves showing in *Colpidium* death following inoculation according to the age of the parent culture, which is written over each curve.

a one day old parent produces 38·4 in 24 hours, a two day parent 5·9, a three day parent 2·6 and a four day parent 2·0. In the case of the one day old parent, where he obtains the startling reproductive rate of 5·26 in 24 hours, he cites 27 cases in which the number of progeny produced vary from 11–105.

It is possible that in the case of the initial death the transfer of cells from a stale medium to a fresh one may involve too violent a change in the physical and chemical conditions, and that death is due to these causes. Certain facts, however, militate against such a suggestion. For instance, in the series of experiments with *Colpidium* (see Fig. 5) there is death on inoculation in both

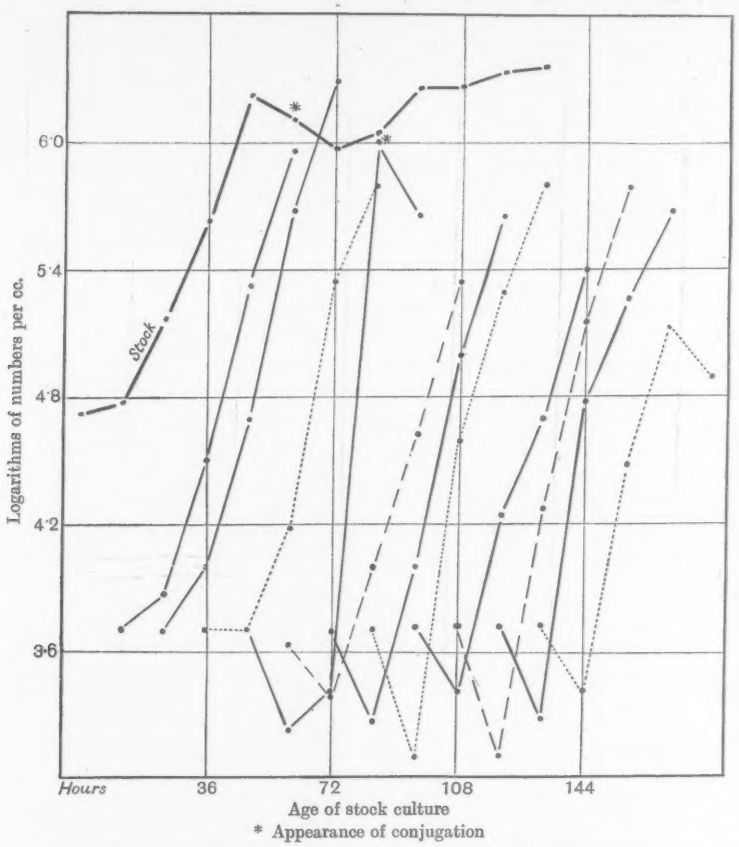

Fig. 6. Curve showing in *Oicomonas* death following inoculation according to the age of the parent culture. The asterisks mark the points where conjugation forms were first seen.

sub-cultures made from the 96 hour parent, but not in those made at 108 hours. It is interesting to notice that the stock culture decreased in numbers between 96 and 108 hours, but between 108 and 120 rose suddenly, which suggests that the initial rise in numbers in the 108 hour sub-culture was due to something inherent in the animals, which the change in medium is powerless to stop.

A further attempt has been made to clear up this point by sub-culturing

from the stock at various ages into its own fluid which is previously filtered. In every case there were very much lower numbers in the filtered liquid than in the fresh medium, but the results are vitiated by the fact that fresh medium when filtered gives lower numbers than control unfiltered medium, indicating that filtration changes the medium too much to permit of any deductions being drawn from such experiments.

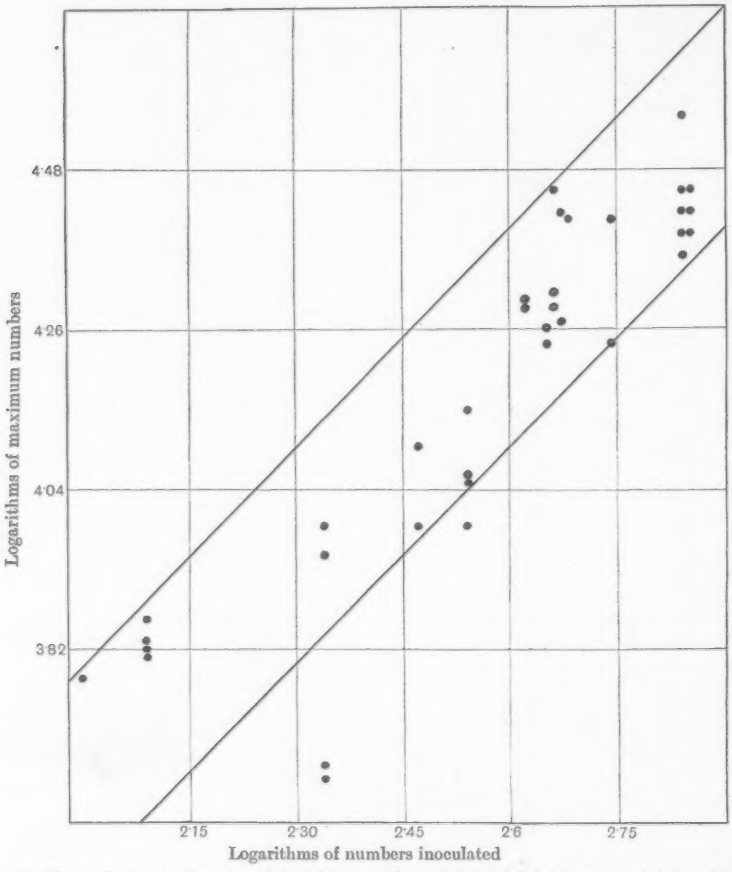

Fig. 7. Figure demonstrating the relation between the numbers of *Colpidium* inoculated and the maxima attained.

Constant reproductive rates.

Old age in the parent culture also influences the later growth of the sub-cultures (Fig. 5), the maximum numbers in such cultures tending to remain very much lower than they are in those from young parents. These lower

numbers which are obtained from old parents are undoubtedly in part due to the impaired vitality to which Calkins draws attention, but the initial death, by reducing the number from which the organisms have to rise, will also tend to keep the final numbers low. The number of organisms per cc. with which a culture starts, appears to influence it throughout its existence to a marked extent. In Fig. 7 the logs. of the numbers of animals inoculated are plotted against the logs. of the maximum numbers attained in the same culture. Each point therefore represents the average number of times that each animal has divided. In 91 % cases the points fall between the lines which include from 5 to 6 divisions.

The highest number ever reached by any of these cultures was about 35,000 animals per cc., but in other experiments there have been as many as 64,000 colpidia per cc.; therefore the comparatively low numbers reached in some cases cannot be in any way due to the environment itself, as is evident by the fact that young rapidly divided animals inoculated into such media are not killed as they would have been had the medium been toxic.

There is a great degree of variation in the size of the inoculum (100–700 per cc.), the age of the parent culture (24–48 hours) and the time taken in reaching the maximum number (48–168 hours) in the cultures represented. It is further of interest that the reproductive rate during the first 24 hours is very varied and apparently bears no relation to the final numbers attained.

Conclusions.

When the work now in progress on single cell cultures has been extended it is hoped to discuss in detail the experiments described above with especial reference to their bearing on the results already obtained from the investigation of protozoa living in the soil.

For the moment it is sufficient to point out how important it is that the experimental methods employed should be standardised if comparative results are to be obtained. To the physiologist this is self evident, but, judging by the papers already published, it has not been sufficiently appreciated by students of micro-biology. Also, to gain an insight into the life cycle of any species of protozoa, it is necessary to make observations, not only over a long period of time, but at frequent intervals. The three hourly count experiment recorded above shows how much, that is probably highly significant, is lost if the cultures are left unattended for even 24 hours. Finally it cannot be too strongly urged that in further work of this nature at least three, and preferably five, parallels to each experiment should be put up.

As regards the actual results obtained it would be premature to attempt an explanation of such phenomena as death following inoculation according to the age of the parent culture, and the constant reproductive rate of certain strains of *Colpidia*.

If in bacterial cultures a connection between the age of the parent and

death following inoculation also obtains it has an important bearing on the method sometimes used of differentiating between strains according to the time taken to ferment certain sugars.

In conclusion we wish to express our thanks to Dr Peters for providing us with the cultures of *Colpidium*, and to Mlle. Perey and Mr Sandon for valuable help during the short period counts.

Summary.

1. Methods are given by which it has been found possible to obtain comparable results when studying the reproductive rates of certain protozoa in mass cultures.

2. It is shown that within a relatively short period after inoculation, under certain conditions, a varying proportion of the organisms die; and that this is correlated with the age of the culture from which the inoculation was made.

3. By means of three hourly counts it was found that death occurs even during the period of maximum reproduction.

4. Evidence is supplied that in certain strains of *Colpidium* the rate of reproduction from inoculation to the maximum numbers attained is constant.

References.

Calkins (1919). *J. Exp. Zool.* **29**, 121.
Cutler, Crump, Sandon (1922). *Roy. Soc. Phil. Trans.* B. **211**, 317.
Dale (1913). *J. Physiol.* **46**, 129.
Hopkins, H. S. (1922). *J. Exp. Zool.* **34**, 339.
Hutchison (1913). *J. Exp. Zool.* **15**, 131.
Jennings (1920). Life and Death, Heredity and Evolution in Uni-Cellular Organisms.
Jollos, V. (1913). *Biol. Centr.* **33**, 222.
McKendrick and Kesava Pai (1911). *Proc. Roy. Soc. Edin.* **31**, 649.
Penfold (1914). *J. Hygiene*, **14**, 215.
Peters (1921). *J. Physiol.* **55**, 1.
Robertson (1921). *Biochem. J.* **15**, 595.
Student (1907). *Biometrika*, **5**, 351.
Wilson (1922). *J. Bact.* **7**, 405.

XXIV. INFLUENCE OF A MILK DIET ON THE SKELETON.

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Rickets is developed principally at the age when milk forms the staple diet. Most authors (Aron [1908], Schabad [1910], Dibbelt [1910], Korenchevsky [1922, 3]) draw attention to the fact that a child fed on a milk diet is liable to suffer from calcium starvation, which is an important factor in the development of rickets. Pfeiffer [1885] found that the milk of mothers of rickety children might be less rich in phosphorus. According to the investigations of McCollum and co-workers [1921, 1922] this deficiency, in conjunction with the deficiency of a fat-soluble factor, may be one of the causes of rickets. Daniels and Loughlin's experiments [1920], if confirmed by experiments on a greater number of animals, might explain the cause of the decreased content of salts in the milk: these two authors found that pasteurisation and prolonged heating caused the loss of calcium salts from milk. Rats fed on such milk did not grow, and even perished from exhaustion. The addition of calcium salts (especially tricalcium phosphate or calcium glycero-phosphate) was followed by a resumption of normal growth. This result could not be obtained by adding vitamin A or B to the milk.

The work of Mellanby [1919, 1920, 1921] and Korenchevsky [1921, 1922, 1, 2, 3; 1923, 1, 2] showed the importance of a deficiency only of a fat-soluble factor in the production of rickets, and Korenchevsky's investigations showed the importance of the simultaneous deficiency of the fat-soluble factor *plus* calcium. In the present work, "fat-soluble factor" is applied to a factor or factors connected chiefly with animal fats, and conducing to the normal metabolism of calcium in the organism, to growth, nutrition, and appetite in animals. We consider "fat-soluble factor" a suitable term in view of the fact that the existence of a fourth, anti-rachitic, vitamin, apart from vitamin A [McCollum and co-workers, 1921, 1922], has not yet been fully proved. The existence, in milk, of a fat-soluble factor possessing anti-rachitic properties raises the question of its possible deficiency in some milk, and therefore, of the possibility of a milk diet producing rickets in consequence of this. The investigations of Hughes, Fitch and Cave [1921], Drummond, Coward and Watson [1921], Dutcher [1921], Kennedy and Dutcher [1922] showed that the content of vitamin A in cow's milk or in butter made from it may fluctuate

very greatly, principally in dependence on the vitamin content in the food of the cow.

In making butter, part of the vitamin A contained in the milk is inactivated [Drummond, Coward and Watson, 1921]. In some samples of butter these authors found a surprisingly small content of vitamin A. On the other hand, Hopkins [1912, 1920] found that the addition of 2 cc. of fresh milk to the food deprived of vitamins was enough to maintain the normal growth and health of rats, that is to say, some kinds of milk are really very rich in vitamins. The factor acting destructively on vitamin A is oxidation, especially on heating, but not heating alone [Hopkins, 1920; Drummond and Coward, 1920; Zilva, 1920; Mellanby, 1921; McCollum and co-workers, 1922].

In the present investigation we shall not quote all the literature on the influence of fresh, heated or desiccated milk on the growth, nutrition and reproduction of animals. Such literature has been collected and properly compared in the works of Mattill and Conclin [1920], and Daniels and Loughlin [1920]. In the summary of their investigation Mattill and Conclin give a clear idea of the present position regarding this question. In their experiments, young rats after weaning were placed on various rations, consisting primarily of cow's milk, fresh and desiccated. On fresh milk rats made good initial growth, but beginning between the 50th and 100th day of life, especially in the females, there was a decided retardation. There was no reproduction. The addition of yeast filtrate temporarily increased the rate of growth, and one female became pregnant, but the litter was eaten. Addition of wheat embryo also increased the rate of growth, with no further effect thus far. When milk was supplemented with iron citrate, growth was much more satisfactory, but reproduction was not successful. The growth failure was in part the result of the dilute form of the food, for on dry milk rats made much better growth, and their average food consumption in milk solids was considerably greater than that of those fed on fresh milk. The females, however, remained somewhat below normal after 75 days, and again there was no reproduction. Neither substitution of dry skim milk to which butter fat was added equivalent to that in dry whole milk, nor the addition of 10 % of butter fat to dry whole milk, was successful.

On a ration containing 55 % of dry whole milk, 40 % of starch, and 5 % of butter fat, both male and female rats made practically normal growth, and the females bore young, but did not rear them. A similar ration has been used by other investigators, who secured not only satisfactory growth, but also reproduction, while others again have, like ourselves, not had success with whole dry milk alone. Dilution of dry milk by 25 % lactose resulted in poor growth and no reproduction. The testes were, with one exception, of normal weight, and contained motile spermatozoa. The ovaries, on the other hand, were much under normal weight, even 50 % and more. In Mattill and Conclin's opinion, possibly milk is lacking both quantitatively and qualitatively in substances necessary for successful adolescent growth

and reproduction, especially in the female, and it may contain substances that are inhibitory to the growth of an animal in the third or mature growth cycle. A ration containing dry milk and 1 % of yeast is the only one on which normal growth and partially successful reproduction were obtained, and the growth of rats on this ration, and on one containing 5 % of yeast, is thus far practically normal. Whether yeast supplies something unique remains to be seen.

As far as we know, there have been only three investigations of the influence of a milk diet on the skeleton. Bolle [see Bartenstein, 1905] found that milk, either fresh or boiled for five minutes, on which guinea-pigs were fed almost exclusively, produced no skeletal changes in the latter. Milk boiled for 10 or 20 minutes produced osteoporosis, fragility of the bones and a partial separation of ephiphyses in the skeleton of guinea-pigs. The skeletons were not examined either microscopically or chemically.

Keller [see Bartenstein, 1905] obtained negative results on repeating Bolle's experiments on mice and dogs.

Bartenstein [1905], on the contrary, obtained, on the whole, the same results as Bolle. In his experiments, the daily ration of the guinea-pigs consisted of milk *ad libitum*, with the addition of cream (up to 8–12 % fat), 2 g. of hay and 1 g. of wheat bran. Unfortunately, for the purpose of histological examination the bones were completely decalcified in H_2SO_4, and therefore the question of the presence of rickets could not be solved. The author considers the resultant histological picture analogous to the osteotabes infantum of Ziegler [1901].

Thus, up to the present time there have been no investigations, carried on in accordance with modern technical requirements, for the purpose of determining the influence of a milk diet on the skeleton. The object of the present investigation was to ascertain the influence on the skeleton of rats of diets consisting of various doses of fresh, heated and oxidised milk. Oxidation was attempted for the purpose of diminishing, or destroying, its antirachitic properties in order to produce rickets in the rats by such a diet. In all there were three series of experiments. The object of the first series was to study the effects of large doses of milk; that of the second series—the effects of medium doses, and of the third series—of small doses. The histological and chemical methods of investigating the skeleton were the same as those used in previous investigations [Korenchevsky, 1921, 1922].

First series of experiments. Large doses of milk.

Seven experiments were conducted on 51 rats, milk being given *ad libitum*. 3 % of cane sugar was added to the milk, in order to increase its calorie content. The milk was supplied by a large London firm, and was a mixture of milk from different cows. The experiment lasted from December to 10th March, *i.e.* "winter" milk was used.

The milk supplied was divided into three parts: one was left fresh (FM), the second was heated in a bath of boiling water in a flask for seven hours (HM); the third (OM) was aerated by a brisk stream of air and at the same time heated in a bath of boiling water for seven hours, for the purpose of oxidising the fat-soluble factor. Before being passed through the milk, the air was heated in a coil, likewise immersed in boiling water. Owing to this the temperature of the milk during oxidation averaged 98°·8 C. Milk thus oxidised was used in experiments Nos. 36–42 of this series.

The calcium content of the milk when boiled for ten minutes, heated in a bath of boiling water, with or without aeration, was hardly altered, as shown by our analysis of nine samples of milk. The average figures were as follows:

(1) Fresh milk ... ... 0·122 % Ca
(2) Boiled for ten minutes ... ... 0·123 % Ca
(3) Heated for seven hours in a bath of boiling water 0·121 % Ca
(4) Oxidised and heated for seven hours ... ... 0·113 % Ca

That is to say, only the oxidised milk showed a slight decrease (about 7 %) below the normal in its calcium content. The milk itself became thicker on aeration: this was not due to the evaporation of water, as a reflux condenser was placed over the flask during oxidation for the purpose of preventing evaporation. In order to make sure that the resulting skeletal changes on a milk diet were due solely to the changes in the amount of the fat-soluble factor, and not to that of vitamins B and C, each rat received daily, over and above milk with 3 % sugar, 3 g. of BC 1 paste, composed of the following:

Paste BC 1:	Starch ...	57 g.
	Yeast ...	11·4 g.
	Orange juice	13·6 cc.
	Oxidised milk	18 cc.

For control purposes, during the last third or half of the period of milk diet, some of the rats were given, in addition to paste BC 1, a daily additional 0·03 g. cod-liver oil (paste ABC), or, in some experiments, the control rats were given "normal paste" containing about 1·7 % cod-liver oil[1].

Milk was given to the rats *ad libitum*; their average consumption was as follows:

Table I.

	Beginning of experiment cc.	End of experiment cc.
Fresh milk	37	50
Heated milk	35	70
Oxidised milk	35	58
„ „ +ABC paste	35	56

That is to say, on the whole, the rats consumed a slightly larger quantity of heated, and even oxidised milk than of fresh milk. The very high average

[1] The composition of N paste was the same as that used by Korenchevsky [1921, 1922].

figure of heated milk consumed may be explained by the fact that this average was obtained from three rats, the rest of the rats of this litter also consuming more than the average of fresh or oxidised milk shown in Table I (65 cc. and 63 cc. respectively). The average amount of "normal" paste consumed by the rats was about 20 g.

In the various experiments of the first series the rats were divided into the following groups (see Table II):

Table II. *Diet of rats.*

No. of experiment	FM + 3 g. BC 1	HM + 3 g. BC 1	OM + 3 g. BC 1	OM + 3 g. ABC	"Normal" paste
36	1	—	3	1	1
37	2	—	3	2	2
38	1	—	2	—	1
39	2	—	4	—	1
40	1	1	2	—	1
41	1	2	2	1	1
58	7	—	—	—	1
Total	15	3	16	4	8

In every experiment the rats were taken from the same litter. The rats in experiment 58 were born of parents fed on FM diet, and were themselves fed on that diet after weaning.

Besides these experiments, this series includes one more experiment, No. 42, conducted on five rats, described later.

The average figures of the age of the rats, of the duration of the milk diet, as also the average chemical composition of the skeleton of the rats in experiments 36 to 41, are shown in Table III. As the results were practically identical, no tables of separate experiments are given.

Table III. *Chemical composition of the skeleton of rats fed on milk ad libitum.*

No. of experiment	Age in days		Feeding in days	FM + BC 1 in bones			HM + BC 1 in bones			OM + BC 1 in bones			OM + N paste in bones			OM + ABC in bones		
	Initial	Final		H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %
Exp. 40 (5 rats)	23	66	43	42·0	12·1	20·8	38·7	13·2	21·6	37·7	13·6	21·9	37·9	13·4	21·5	—	—	—
„ 38 (4 rats)	17	105	88	39·7	13·2	21·9	—	—	—	37·3	13·6	21·7	37·9	14·2	22·7	—	—	—
„ 41 (7 rats)	24	109	85	36·7	13·9	22·0	36·5	14·1	22·0	37·3	13·9	22·1	39·7	12·9	21·3	39·4	12·6	20·7
„ 36 (6 rats)	21	118	97	37·6	13·9	22·3	—	—	—	37·6	14·1	22·7	39·4	13·0	21·5	36·7	14·5	22·5
Average of exps. 38, 41, 36	21	111	90	38·0	13·7	22·1	36·5	14·1	22·0	37·3	13·9	22·2	39·0	13·4	21·8	38·0	13·5	21·6
Exp. 37 (9 rats)	36	130	94	38·7	14·0	22·9	—	—	—	36·6	14·4	22·7	38·2	14·0	22·6	37·9	14·2	22·8
„ 39 (7 rats)	40	140	100	37·7	14·1	22·6	—	—	—	38·3	13·6	22·2	40·3	12·8	21·5	—	—	—
Average of exps. 37, 39	38	135	97	38·2	14·1	22·7	—	—	—	37·5	14·0	22·5	39·2	13·4	22·1	37·9	14·2	22·8

These data show that in the different groups of rats consuming milk *ad libitum* no essential difference in the chemical composition of the skeleton was observed, even when, for instance, the oxidised milk diet was begun at a very early age, immediately after weaning (17th day after birth), and was continued for about three months.

Histological examination likewise showed no difference between the structure of the skeleton of rats fed on FM, HM or OM. Sometimes the microscopical picture of the skeleton of rats on a milk diet differed from that of rats on a normal diet only in being slightly more osteoporotic, this condition being more perceptible in rats fed on OM. The osteoporosis was due to the lowered activity of osteoblasts (the number of osteoclasts being normal).

The weight curves of rats of various categories in experiments 36–41 *were identical, and in no way different from the weight curves of rats on a normal diet.* In view of this, they are not shown here. There was likewise no difference in the external appearance and nutrition of the various groups of rats.

Thus the large ration of OM consumed by the rats retained sufficient of the fat-soluble factor for the growth of the rats, and the composition and structure of their skeleton were practically normal even when air was passed through the milk at a temperature of 98°·8 C. during seven hours.

In experiment 58, the second generation of rats on FM diet showed a marked difference from the normal as regards external appearance: rats killed at the age of 57 days were some 60 % below the normal weight; in many places the fur was so thin that some rats were nearly hairless. Moreover, they all suffered from emaciation and anaemia. Chemically, the skeleton was normal in structure. Histologically, the bones showed a moderate increase of the proliferating cartilage with an insufficient deposition of calcium into the zone of provisional calcification in the case of some rats. There was considerable osteoporosis, but the amount of osteoid was not increased.

The object of experiment 42 was to ascertain the influence on the skeleton of rats of an addition to the milk diet either of vitamin B alone (in the form of a paste with yeast), or of vitamin C alone (paste with orange juice). The milk given to the rats was either fresh or oxidised.

Neither the chemical nor the histological examination of the skeleton showed any effects of the addition of vitamins B and C to the milk given to the rats *ad libitum*. The composition of the skeleton of the rats in experiment 42 was compared with that of the control rats from the same litter, fed on a "normal" diet. In view of the negative results of the experiment, we give no detailed data.

Second series of experiments. Influence of medium doses of milk (15 to 30 cc. per day).

Thirty-nine rats, belonging to six litters, were used in this series of experiments. The milk was given to the rats in the same form as in the first series of experiments, *i.e.* FM, HM and OM; except that, for the purpose of intensifying the process of the oxidation of the fat-soluble factor, 1 cc. of a 20 % solution of hydrogen peroxide was added per 100 cc. milk. On investigation it was found that after heating the milk for seven hours and passing air through it, any reaction of hydrogen peroxide had disappeared.

The experiments were conducted from February to the first half of May, *i.e.* during the first third or half of the experimental period "winter" milk was used, and the rest of the time "spring" milk.

In this series of experiments the young rats were weaned at the age of 19 to 20 days, after which, for a period of from 13 to 34 days (Period I), they were fed exclusively on OM. From the age of 33–35 days the rats were transferred to a diet consisting of a moderate ration of milk (15 to 30 cc.) (Period II). The number of calories taken in by each rat was equalised by the starch given to the rats in pastes BC II and BC III. In these pastes the rats received approximately the same amount of vitamins B and C in the daily dose. The composition of the paste was as follows:

	Paste BC II	Paste BC III
Starch	61·5 g.	66 g.
Yeast	3·5 g.	5 g.
Orange juice	5·0 cc.	6 cc.
Water	30·0 cc.	23 cc.

To the daily ration of 15 cc. milk was added 16 g. of paste BC II; to 30 cc. milk 11 g. of paste BC III.

For purposes of comparison some rats received, even during Period II, large doses of milk with 3 g. of paste BC I, or 3 g. of paste ABC (see p. 190).

During Period II the rats were fed for 86 to 103 days, after which they were killed at ages varying from 122 to 151 days.

Thus, during Period II, the rats in the experiments of the second series, as compared with those of the first series, received a considerably smaller amount of protein, fat, salts, and fat-soluble factor.

In the various experiments of the second series the rats were divided into the following groups (see Table IV):

Table IV. *Diet of rats during Period II.*

No. of exp.	50 cc. FM +3 g. BC I	15 cc. FM +16 g. BC II	50 cc. OM +3 g. BC II	50 cc. OM +3 g. ABC	15 cc. OM +16 g. BC II	30 cc. OM +11 g. BC III	30 cc. OM +11 g. BC III + 0·03 g. cod-liver oil
52	—	2	—	1	4	—	—
53	1	2	—	1	3	—	—
54	—	—	—	—	2	2	2
56	1	2	1	—	2	—	—
57	1	2	1	—	2	—	—
Total	3	8	2	2	13	2	2

Besides this, in experiment 55 there were seven rats the distribution of which will be mentioned later, in describing that experiment.

In experiments 52, 53, 54, 56 and 57, during Period I, *i.e.* that of an exclusive diet of OM, the first few weeks after weaning the rats consumed, on an average, about 23 cc. of milk per diem, and later about 40 cc. Those rats which received milk *ad libitum* during the whole experimental period, consumed about 50 cc. during the last third of the time, there being no difference in the consumption of FM and OM. The ration of 15 to 30 cc. of

milk and pastes BC II and BC III was completely consumed by the rats, with the exception of one rat, fed on OM.

There was very little difference in the appearance of the rats of each group. The curves of the average weight of the rats of each group in these experiments are shown in Fig. 1 (males) and Fig. 2 (females). The weight curves of rats on OM are somewhat below those of rats on FM, this being more marked in the case of rats on a ration of 15 cc. milk. A similar, merely slight, retardation of the weight curves was observed in the case of rats fed on 15 cc. milk, as compared with those of rats receiving and consuming about 50 cc. milk. The age, duration of feeding on special diets, and the chemical composition of the skeleton of rats in the experiments described are given in Table V. This table shows the following:

Table V. *Chemical composition of the skeleton of rats fed on 15–50 cc. of milk.*

	Age in days			Duration of feeding in days		50 cc. FM+ABC in bones			50 cc. FM+BC I in bones			50 cc. OM+ABC in bones			50 cc. OM+BC I in bones			15 cc. FM+BC II in bones			15 cc. OM+BC II in bones		
No. of exp.	When put on OM only	When put on milk and paste	Final	On OM only	On milk and paste	H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %
Exp. 52 (7 rats)	19	53	146	34	93	—	—	—	—	—	—	35·2	14·7	22·6	—	—	—	35·1	13·7	21·1	36·0	13·3	20·8
Exp. 53 (7 rats)	19	54	151	35	97	31·4	16·3	23·7	—	—	—	32·5	14·6	21·7	—	—	—	32·7	14·7	21·9	35·3	14·5	21·9
Exp. 54 (2 rats)	20	45	144	25	99	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	39·9	12·1	20·1
Average of exps. 52 and 53	19	54	149	35	95	31·4	16·3	23·7	—	—	—	33·8	14·7	22·2	—	—	—	33·9	14·2	21·5	37·1	13·3	20·7
Exp. 56 (6 rats)	20	33	133	13	100	—	—	—	32·6	15·9	23·7	—	—	—	32·1	14·9	22·0	35·4	13·7	21·2	34·4	14·0	21·6
Exp. 57 (6 rats)	20	33	136	13	103	—	—	—	30·5	15·5	22·4	—	—	—	34·1	13·8	20·9	34·1	14·1	21·3	38·9	12·7	20·8
Average of exps. 56 and 57	20	33	135	13	102	—	—	—	31·6	15·7	23·0	—	—	—	33·1	14·3	21·4	34·7	13·9	21·3	36·6	13·4	21·1

On an OM diet, a slight diminution of calcium in the skeleton was observed: *e.g.* on 50 cc. OM the skeleton, as compared with that of rats on FM, was more deficient in dry bone (about 7 %) and in fresh bone (9 %); on a diet of 15 cc. milk it was not in every experiment that even this slight difference was observed. In any case, the difference seen in the composition of the skeleton was so slight that it might have been due to physiological fluctuations. Histologically, there was also no considerable difference between the structure of the skeleton of rats in different groups. Only a slight degree of osteoporosis might be observed in the rats especially fed on OM.

Experiment 55 was conducted in such a manner as to approximate the conditions to those of bringing up children by hand, that is to say, the rats were given diluted milk *ad libitum*. The requisite number of calories was provided by a 17 % solution of cane sugar, with which the milk was diluted. The milk was heated in a bath of boiling water for seven hours. The seven

rats used in this experiment were divided into four groups, and at the age of 36 days were placed on the following diets:

Table VI.

Denomination of milk	No. of rats	Dilution of milk: Percentage of 17 % solution of cane sugar contained in milk	Consumption of milk in cc. Beginning of experiment	Consumption of milk in cc. End of experiment
HM	2	—	27	46
DM_1	2	60	22	50
DM_2	2	40	23	50
DM_3	1	50	24	28

Besides milk, each rat received 3 g. of paste BC 1. After 86 days of feeding on the above-mentioned diets, the rats were killed. The rats fed on DM_1 and DM_2 were slightly (about 8 %) lighter in weight than those on HM, but, on the whole, were not different from them in any other respect. The rat on DM_3 weighed 37 % less than the rats on HM, and was thin. As may be seen from Table VI, its appetite was worse than that of any of the others.

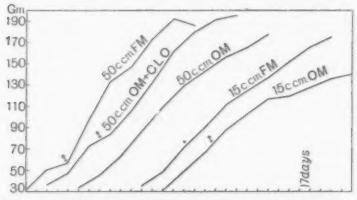


Fig. 1. Weight curves of average rats (males) from experiments 52, 53, 54, 56 and 57. Up to the point shown approximately by the arrows rats were kept on OM only. After that the amount and kind of milk are shown above each curve. C.L.O. = cod-liver oil.

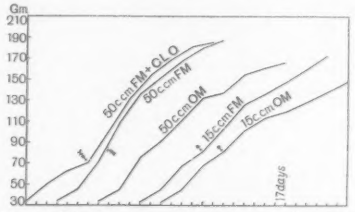


Fig. 2. Weight curves of average rats (females) from experiments 52, 53, 54, 56 and 57. Up to the point shown approximately by the arrows rats were kept on OM only. After that the amount and kind of milk are shown above each curve. C.L.O. = cod-liver oil.

In all the rats fed on diluted milk, as compared with the rats fed on HM, the composition of the skeleton was worse: the average water content of the bones was 8 % greater, the calcium content in fresh bone was about 15 % less, and about 12 % less in dry bone. Histologically, the rats fed on diluted milk, as compared with those fed on HM, showed more marked osteoporosis, and in some cases there was a slight enlargement of cartilage and an irregular line of osseo-chondral junctions. A slightly increased amount of osteoid was observed only in the case of one rat fed on DM_3.

Thus, in this series of experiments also, no considerable skeletal changes could be obtained. It is a striking fact that the seven-hours' aeration of milk at 98°·8 C. did not diminish the amount of the fat-soluble factor in the milk to such an extent that the growth of the rats and the calcification of their skeleton was affected considerably, even by the ingestion of only 15 cc. of milk per diem. On the other hand, larger quantities of milk (up to 30 cc.), diluted with 17 % sugar solution, caused greater skeletal changes, that is to say, apparently an exclusively *liquid* diet has an unfavourable effect on animals if continued for a long time.

THIRD SERIES OF EXPERIMENTS. INFLUENCE OF SMALL DOSES OF MILK (5 CC.) ON THE SKELETON OF RATS FED ON A DIET DEFICIENT IN THE FAT-SOLUBLE FACTOR.

In this series there were 40 rats, belonging to five litters. In each experiment the rats, after being put on a diet deficient in the fat-soluble factor (− A diet)[1] for 33–39 days, were divided into the following groups:

Group I remained on −A diet;
„ II transferred to normal diet (N)[1], containing butter and cod-liver oil as a source of fat-soluble factor;
„ III fed on −A diet + 5 cc. FM;
„ IV fed on −A diet + 5 cc. HM; and
„ V fed on −A diet + 5 cc. OM.

The distribution of the rats into various groups in each experiment, their weight, age, duration of various dieting, the chemical composition of their skeleton and the histological diagnosis, are shown in Tables VII–XI.

The amount of N paste consumed by the rats fluctuated between 16 and 21 g.; the amount of −A paste consumed per diem varied in the different experiments: in experiments 43 and 44 from 10 to 14 g. were eaten; in experiment 45 from 9 to 11 g., but in the case of rats fed on FM and of one (490) fed on HM, the paste was consumed in larger quantities—14 to 15 g.; in experiment 47 the average consumption reached 10 g.; in rats fed on FM it was 11 g. Thus with some rats the ingestion of fresh milk alone increased their appetite.

Table VII.

Experiment 43. The influence of 5 cc. of milk on the chemical composition of the skeleton of rats kept on −A diet without milk 38 days (period I), with milk 20 days (period II). Final age: 96 days.

			Weight in g.			In bones			
No. of rat	Sex	Diet in period II	When put on −A diet	At the beginning of period II	Final	H_2O %	Fresh Ca %	Dry Ca %	Histological results
474	♂	N + FM	43	58	132	38·9	13·0	21·2	*Normal
477	♀	−A + FM	51	70	97	47·8	8·7	16·6	Osteoporosis
480	♂	−A + FM	46	74	95	50·0	7·5	15·1	Slight rickets and osteoporosis
		Average of 477 and 480:—				48·9	8·1	15·9	
475	♀	−A + HM	56	73	98	46·2	8·4	15·5	Slight osteomalacia
476	♂	−A + OM	55	75	92	48·0	7·7	14·7	Slight osteomalacia
479	♂	−A + OM	48	64	87	47·5	7·3	14·4	Slight rickets
		Average of 476 and 479:—				47·7	7·5	14·6	
478	♂	−A alone	53	84	87	51·3	7·5	15·4	Slight rickets. Osteoporosis. Died from cachexia 3 days before the end of the experiment

* Analyses lost; these figures of the chemical composition are taken from rat 501 which was of a corresponding age. (See also very similar figures from rats aged 80–120 days [Korenchevsky, 1922, 3, p. 65].)

[1] For the composition of the diets N and −A, see previous communications [Korenchevsky, 1921, 1922].

Table VIII.

Experiment 48. The influence of 5 cc. of milk on the chemical composition of the skeleton of rats kept on −A diet without milk 33 days (period I), with milk 26 days (period II). Final age: 104 days.

No. of rat	Sex	Diet in period II	Weight in g. When put on −A diet	Weight in g. At the beginning of period II	Weight in g. Final	In bones H_2O %	In bones Fresh Ca %	In bones Dry Ca %	Histological results
510	♀	−A+FM	62	72	110	43·5	9·8	17·3	Slight rickets
513	♂	−A+FM	62	78	132	49·4	8·3	16·5	Slight rickets, osteoporosis
		Average of 510 and 513:—				46·5	9·1	16·9	
507	♀	−A+HM	69	60	89	48·1	8·5	16·5	Slight rickets, osteoporosis
511	♀	−A+HM	85	96	128	42·9	10·4	18·3	Slight rickets
		Average of 507 and 511:—				45·5	9·5	17·4	
508	♀	−A+OM	63	72	87	48·1	8·1	15·6	Slight rickets, osteoporosis
512	♂	−A+OM	88	120	144	45·5	9·5	17·4	Slight rickets
		Average of 508 and 512:—				46·8	8·8	16·5	
509	♂	−A alone	85	112	128	45·7	8·4	15·5	Moderate rickets

Table IX.

Experiment 47. The influence of 5 cc. of milk on the chemical composition of the skeleton of rats kept on −A diet without milk 39 days (period I) with milk 48 days (period II). Final age: 109 days.

501	♀	N+FM	47	59	158	38·9	13·0	21·2	Normal
502	♂	−A+FM	49	70	113	51·0	8·6	17·5	Very slight rickets
506	♂	−A+FM	56	74	137	49·2	8·5	16·7	Slight rickets
		Average of 502 and 506:—				50·1	8·6	17·1	
504	♂	−A+HM	57	70	118	50·4	6·9	13·8	Slight rickets, osteoporosis
500	♂	−A+OM	54	70	117	53·3	6·6	14·0	Moderate rickets
503	♀	−A+OM	41	63	65	56·7	6·7	15·6	Slight rickets. Osteoporosis. Hyperaemia of bone marrow. Died from cachexia
		Average of 500 and 503:—				55·0	6·7	14·8	
505	♀	−A alone	43	62	81	49·5	7·6	15·0	Moderate rickets

Table X.

Experiment 45. The influence of 5 cc. of milk on the chemical composition of the skeleton of rats kept on −A diet without milk 35 days (period I), with milk 63 days (period II). Final age: 139 days.

493	♀	N+FM	35	55	180	38·1	13·3	21·5	Normal
494	♂	N+FM	36	60	248	44·3	11·7	21·0	Normal
		Average of 493 and 494:—				41·2	12·5	21·2	
485	♀	−A+FM	43	79	106	41·2	11·2	19·0	Slight rickets
488	♂	−A+FM	32	68	163	45·2	8·9	16·2	Slight rickets
		Average of 485 and 488:—				43·2	10·0	17·6	
486	♀	−A+HM	34	58	103	49·0	7·8	15·3	Slight rickets
490	♂	−A+HM	34	75	162	46·7	8·3	15·7	Slight rickets
		Average of 486 and 490:—				47·8	8·1	15·5	
484	♀	−A+OM	35	65	94	44·5	8·0	14·3	Slight rickets
491	♂	−A+OM	33	74	125	44·0	7·3	13·1	Slight rickets, osteoporosis
492	♀	−A+OM	37	62	99	50·4	7·5	15·0	Moderate rickets
		Average of 484, 491 and 492:—				46·3	7·6	14·2	
487	♂	−A alone	35	71	82	50·9	6·4	13·1	Severe rickets
495	♂	−A alone	47	86	117	47·6	7·3	14·0	Moderate rickets, osteoporosis
496	?	−A alone	56	84	122	49·9	6·9	13·9	Severe rickets
		Average of 487, 495 and 496:—				49·5	6·9	13·6	

Table XI.

Experiment 44. The influence of 5 cc. of milk on the chemical composition of the skeleton of rats kept on −A diet without milk 38 days (period I), with milk 66 days (period II). Final age: 144 days.

No. of rat	Sex	Diet in period II	Weight in g. When put on −A diet	Weight in g. At the beginning of period II	Weight in g. Final	In bones H_2O %	In bones Fresh Ca %	In bones Dry Ca %	Histological results
445	♀	N+FM	57	67	143	40·3	12·2	20·5	Normal
451	♀	−A+FM	49	62	105	43·5	11·2	19·8	Nearly normal
447	♂	−A+HM	67	104	134	44·6	10·1	18·3	Slight rickets
449	♀	−A+HM	58	71	103	41·4	11·0	18·7	Nearly normal
		Average of 447 and 449:—				43·0	10·5	18·5	
448	♂	−A+OM	60	78	151	40·8	10·3	17·6	Slight rickets
450	♂	−A+OM	54	84	133	49·5	8·9	17·7	Slight rickets
		Average of 448 and 450:—				45·2	9·6	17·7	
446	♂	−A alone	66	87	99	47·2	8·8	16·6	Slight rickets. Moderate osteoporosis

The weight curves of rats are given in Figs. 3–6, and show that the addition of 5 cc. milk to −A diet improved the growth of the rats. Moreover, the greatest effect was produced by FM, to a lesser degree by HM, the least effect being produced by OM; that is to say, the mere heating of the milk for seven hours at 100° C. in most cases caused a slight disintegration of the growth-promoting properties of milk, but the greatest effect was produced by oxidation combined with heating. At autopsy, the following peculiarities were observed in the various groups of rats: on N diet the rats were fat and normal; on −A diet they were thin or emaciated, with opaque teeth, frequently with depressed thorax and numerous fractures of the ribs. In some rats the ribs and the bones of their hind legs were curved.

On −A diet + 5 cc. OM the macroscopical changes hardly differed from those on −A diet alone, the only difference being that the rats were somewhat better nourished.

Table XII. *Summary table showing in percentage the influence of small doses (5 cc.) of milk on the chemical composition of the skeleton of rats kept on −A diet.*

No. of exp.	Above (+) or below (−) −A standard. In bones: N+FM H_2O	N+FM Fresh Ca	N+FM Dry Ca	−A+FM H_2O	−A+FM Fresh Ca	−A+FM Dry Ca	−A+HM H_2O	−A+HM Fresh Ca	−A+HM Dry Ca	−A+OM H_2O	−A+OM Fresh Ca	−A+OM Dry Ca
I. Milk given during a period of 20–26 days (average 23 days).												
Exp. 43 (7 rats)	−24·2	+73·4	+37·5	− 4·7	+ 8·0	+ 2·7	−10·1	+12·0	+ 0·8	− 7·0	− 4·0	−5·6
,, 48 (7 rats)	—	—	—	+ 1·6	+ 7·6	+ 9·0	− 0·5	+12·8	+12·1	+23·6	+ 4·4	+6·5
Average of exps. 43 and 48	−24·2	+73·4	+37·5	− 1·6	+ 7·8	+ 5·9	− 5·3	+12·4	+ 6·5	+ 8·3	+ 0·2	+0·45
II. Milk given during a period of 49–66 days (average 60 days).												
Exp. 47 (7 rats)	−21·3	+70·9	+41·2	+ 1·3	+12·4	+13·7	+ 1·9	− 9·5	− 7·8	+11·2	−12·3	−1·2
,, 45 (12 rats)	−16·7	+81·3	+55·6	−12·6	+45·4	+28·9	− 3·3	+17·1	+13·9	− 6·4	+10·0	+3·4
,, 44 (7 rats)	−14·6	+39·0	+23·4	− 7·8	+27·3	+19·6	− 8·9	+19·8	+11·5	− 4·4	+ 9·6	+6·5
Average of exps. 47, 45 and 44	−17·5	+63·7	+40·1	− 6·3	+28·3	+20·7	− 3·4	+ 9·1	+ 5·9	+ 0·1	+ 2·4	+2·9

On −A diet + 5 cc. FM the macroscopical picture showed a marked difference as compared with −A diet: the rats were considerably better nourished, the skeleton was either normal or, if there were calluses after spontaneous fractures, they were solitary cases, and were far smaller than those in rats on −A diet alone or with OM. On −A diet + 5 cc. HM the macroscopical changes in rats were of a medium character between those of rats fed on OM and FM, being more like the latter. The chemical changes shown in Tables VII–XI are summarised in Table XII. It will be seen from

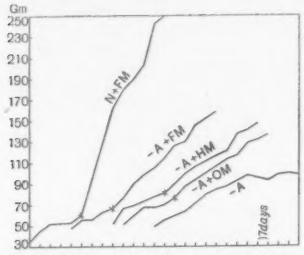


Fig. 3. Weight curves of average rats (males) from experiments 44, 45 and 47. Up to the point marked × rats were kept on −A alone. The diet after that is shown above each curve.

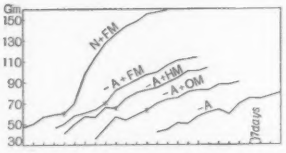


Fig. 4. Weight curves of average rats (females) from experiments 44, 45 and 47. Up to the point marked × rats were kept on −A alone. The diet after that is shown above each curve.

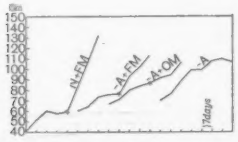


Fig. 5. Weight curves of average rats (males) from experiments 43 and 48. Up to the point marked × rats were kept on −A alone. The diet after that is shown above each curve.

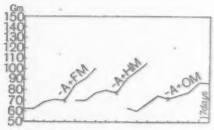


Fig. 6. Weight curves of average rats (females) from experiments 43 and 48. Up to the point marked × rats were kept on −A alone. The diet after that is shown above each curve.

this table that after milk was given for 20–26 days, these changes were only slight, being far more marked after a period of about two months. That is to say, in order for milk to produce a favourable effect, it is necessary that it should be ingested for a considerable period of time. On the whole, the most marked improvement in the chemical composition of the skeleton was observed on giving FM, followed by HM. The addition of OM to −A diet either produced no improvement whatever in the chemical composition of the skeleton or had only a very slight effect. In any case, the addition of 5 cc. milk could not have caused such an improvement in the skeleton in the course of 66 days, as was produced by butter *plus* cod-liver oil in N diet.

On histological examination, the most marked improvement in rickets was observed in the case of a diet of FM or HM. On OM this improvement was usually (though not always) observed, but frequently it was less marked than in the case of FM.

Thus in this series of experiments likewise, the macroscopic, microscopic, and chemical examinations of the skeleton coincided with the data obtained in investigating the growth and weight of animals.

Summary.

In our experiments the rats grew and developed normally, and had a normal skeleton, on a diet of milk *ad libitum* with the addition of the requisite amount of calories in the form of carbohydrates. They were, however, not very fertile and, if they did bear young, the latter became anaemic and cachetic on a milk diet. Though the calcium content of the skeleton of the young was *normal, histologically* there was *osteoporosis*, and even one of the features of rickets (enlargement of the cartilage).

The dilution of heated milk with a 17 % solution of sugar resulted in the composition of the skeleton being worse than that of rats fed on smaller quantities of milk, but with the addition of calories in the form of starch paste. The addition of even a small quantity of milk (5 cc.) to −A diet produced an improvement in rickets caused in rats by a diet deficient in the fat-soluble factor. Such a favourable action of milk was slightly decreased by heating the milk for seven hours at 100° C., and was decreased in a marked degree after the aeration of the milk at 98°·8 C. for the same period of time.

Thus these experiments show that oxidation of milk also reduces its anti-rachitic and growth-promoting properties, as was observed by Hopkins [1920] and Mellanby [1921] in the case of butter. The experiments with larger doses of milk (15 to 50 cc. per diem) have however shown that the oxidation of milk produces *only a partial*, and not total disintegration of the fat-soluble factor, and that in such larger doses oxidised milk produced nearly the same or identical growth of the rats and calcification of the skeleton as was caused by fresh milk. The addition of cod-liver oil to large doses of oxidised milk in most cases had no effect on the results, or if it did affect them, they were only slightly improved.

Thus, in our experiments we failed to obtain such a great disintegration of the fat-soluble factor as to produce rickets in rats fed on an abundant milk diet. No doubt if the oxidation had continued for a longer period, or had taken place at a higher temperature, the results would have been different.

In conclusion, it is necessary to note certain peculiarities obtained on oxidising the fat-soluble factor in milk, as compared with the results of oxidising this factor in butter or cod-liver oil.

Hopkins [1920], Drummond and Coward [1920], and Zilva [1920] showed that the growth-promoting properties of butter are easily disintegrated by oxidation. Mellanby found that oxidation likewise disintegrates the anti-rachitic properties of butter, but not of cod-liver oil. McCollum, Simmonds, Becker and Shipley [1922] found that on oxidising cod-liver oil for 12–20 hours at the temperature of boiling water, its anti-rachitic properties may be preserved,

while its capacity of curing xerophthalmia is lost. On these grounds the American authors have come to the conclusion that the so-called "fat-soluble vitamin A" contains two separate vitamins: (1) an "anti-rachitic vitamin," which is disintegrated with difficulty by oxidation, and (2) "vitamin A" itself, far more easily disintegrated by oxidation, and preserving animals from xerophthalmia.

In our experiments with oxidised milk, the following facts should be noted:

(1) Apparently the fat soluble factor is disintegrated by oxidation with greater difficulty in milk than has been observed in the case of butter. This peculiarity may be to a certain extent explained by the fact that in making butter from milk only part of the growth-promoting factor, present in milk, is transferred to the butter. Thus, according to Drummond, Coward and Watson [1921, p. 544]: "Fresh milk from cows fed on green pasture will, in nearly all cases, induce a resumption of growth in rats fed on a diet deficient in vitamin A, when given in a daily supplement of 2 cc. [cf. Hopkins, 1912, 1920]. This amount of milk may be regarded as supplying approximately 0·1 g. of fat per day. In order to induce the same amount of growth by adding a supplement of butter, it is necessary to give about 0·2–0·3 g. per day."

Certain hypotheses may be advanced with regard to this. For instance, McCollum [1917] thinks that water solutions of the non-lipoid constituents of milk contain this substance (vitamin A), indeed, in considerable amount. His experiments lead him to believe that approximately half of the fat-soluble dietary essential is present in the fat and half in the non-fat portion of the milk. Another hypothesis supposes a partial loss of the fat-soluble factor to occur during the churning of the butter (perhaps due to oxidation). If in milk there is a larger amount of fat-soluble factor than in butter, we assume, that to destroy this factor in milk will take longer and be more difficult.

(2) In our not numerous experiments the mere heating of milk at 100° C. for seven hours almost invariably slightly decreased the growth-promoting and anti-rachitic properties of milk (a phenomenon observable on adding 5 cc. HM to −A diet). This cannot be explained by the separation of lime salts in heating the milk[1]. An investigation of the Ca content in OM did not show any essential alteration in it. Therefore our experiments do not support the hypotheses of Daniels and Loughlin of the decrease in the calcium content of milk as a result of boiling or heating the latter (provided the milk is given to the rats after having been shaken). Finally it may be possible that by heating the milk the calcium compounds are altered to those less able to be assimilated by the organism. However, such a small amount of calcium as is found in 5 cc. of milk and added to −A diet could not influence the composition of the skeleton.

It is known, that merely heating butter or cod-liver oil has no effect on the content of fat-soluble factor in either [Osborne and Mendel, 1915; Hopkins, 1920; Mellanby, 1921]. Therefore, if subsequent and more numerous experi-

[1] The milk was shaken thoroughly before being given to the rats or taken for analyses.

ments confirm the fact noticed by us, it may be found that milk contains, besides a fat-soluble factor, another special factor which to some extent is disintegrated by heating.

(3) In our experiments we did not observe any considerable decrease in the growth-promoting properties of milk, without simultaneous loss of its anti-rachitic properties. Almost invariably there was a parallel decrease in the above-mentioned properties after oxidation. This fact does not agree with McCollum's observations on the relative decrease after oxidation of anti-xerophthalmic and anti-rachitic vitamins in cod-liver oil, though it is in accordance with Mellanby's experiment with butter referred to above.

At present it is difficult to explain this peculiarity, and it is only possible to form conjectures:

(*a*) Taking the point of view of McCollum and co-workers, we may assume that butter contains a small amount of the anti-rachitic factor, while its vitamin A content is large. The great degree of oxidation required to disintegrate the large amount of vitamin A may at the same time disintegrate a small quantity of the anti-rachitic vitamin, *e.g.* in butter. Possibly, the enormous amount of anti-rachitic vitamin contained in cod-liver oil has no time to be inactivated by the degree of oxidation which is sufficient to destroy the anti-xerophthalmic and growth-promoting properties.

(*b*) It is possible that the presence of milk fat in whey, in the form of an emulsion, alters the conditions of the oxidation of the fat-soluble factor.

(*c*) In the same way, this fact may, to a certain extent, be explained by the existence of a special factor in milk, different from the fat-soluble factor.

Conclusions.

(1) When rats are fed on fresh, heated or oxidised milk *ad libitum*, supplemented by carbohydrates, the structure of their skeleton hardly differs from the normal, either chemically or histologically. When oxidised milk is used only a slight degree of osteoporosis in the bones is observed.

(2) Fresh winter milk, as supplied to us by a London Dairy, even to the amount of 5 cc., causes a considerable improvement in rickets induced in rats by −A diet, and a renewal of growth that has been inhibited.

(3) Heating the milk for seven hours at 98·8–100° C., with or without aeration, produces no essential change in its calcium content.

(4) When milk is oxidised at 98°·8 C. for seven hours, its growth-promoting and anti-rachitic properties are obviously decreased, as is seen from experiments with small doses (5 cc.) of milk.

(5) Nevertheless, these properties are so far retained that, on the daily ingestion of 15 cc. and more of oxidised milk by rats for a period up to 132 days, the rats hardly differ, in growth and skeletal development, from those fed on fresh milk.

(6) Apparently, the deficiency of the cow's food in the fat-soluble factor induces a far greater impoverishment of the milk in that factor than the addition of 1 % of peroxide of hydrogen to the milk, and subsequent aeration for seven hours at 98°·8 C.

(7) Heating the milk for seven hours at 100° C. slightly decreases the growth-promoting and anti-rachitic properties of the milk.

A grant from the Medical Research Council and the hospitality of the laboratories of the Lister Institute have enabled us to carry out this work and to them our thanks are due. We wish also to express our sincere gratitude to Professor C. J. Martin for his continuous support in this investigation.

REFERENCES.

Aron, H. (1908). *Biochem. Z.* **8**, 1, 12, 28.
Bartenstein, L. (1905). *J. Kinderheilk.* **61**, 6.
Daniels, A. and Loughlin, R. (1920). *J. Biol. Chem.* **44**, 381.
Dibbelt, W. (1910). *Munch. med. Woch.* **57**, 2121.
Drummond, J. and Coward, K. (1920). *Biochem. J.* **14**, 734.
Drummond, J., Coward, K. and Watson, A. (1921). *Biochem. J.* **15**, 540.
Dutcher, A. (1921). *J. Ind. Eng. Chem.* **13**, 1102.
Hopkins, F. G. (1912). *J. Physiol.* **44**, 425.
—— (1920). *Biochem. J.* **14**, 721, 725.
Hughes, J., Fitch, J. and Cave, H. (1921). *J. Biol. Chem.* **46**, L. (*Proc. Amer. Soc. Biol. Chem.*)
Kennedy, C. and Dutcher, A. (1922). *J. Biol. Chem.* **50**, 339.
Korenchevsky, V. (1921). *Brit. Med. J.* **2**, 547.
—— (1922, 1). *N. York Medical J.* **115**, 612.
—— (1922, 2). *J. Path. Bact.* **25**, 366.
—— (1922, 3). *Spec. Report Ser.* No. 71. Med. Res. Council.
—— (1923, 1). *J. Path. Bact.* **26**, 207.
—— (1923), 2). *J. Path. Bact.* **26**, 222.
Mattill, H. and Conclin, R. (1920). *J. Biol. Chem.* **44**, 137.
McCollum, E. (1917). *J. Amer. Med. Assoc.* **68**, 1379.
McCollum, E., Simmonds, N., Shipley, P. and Park, E. (1921). *J. Biol. Chem.* **47**, 507.
—— —— —— —— (1922). *Johns Hop. Hos. Bull.* **32**, 160.
McCollum, E., Simmonds, N., Becker, E., and Shipley, P. (1922). *J. Biol. Chem.* **53**, 293.
Mellanby, E. (1919). *Lancet*, i, 407.
—— (1920). *Lancet*, i, 856.
—— (1921). *Spec. Report Ser.* No. 61. Med. Res. Council.
Osborne, J. and Mendel, L. (1915). *J. Biol. Chem.* **20**, 379.
Pfeiffer, E. (1885). *J. Kinderheilk.* **24**, 248.
Schabad, I. (1910). *Arch. Kinderheilk.* **52**, 68.
Ziegler, E. (1901). *Centr. allg. Path. path. Anat.* **12**, 865.
Zilva, S. S. (1920). *Biochem. J.* **14**, 740.

XXV. ON THE EFFECT OF DEFICIENCY OF IRON IN THE DIET OF PIGS.

(PRELIMINARY COMMUNICATION.)

By JOHN POOL McGOWAN and ARTHUR CRICHTON.

From the Rowett Research Institute, Aberdeen.

(*Received February 16th, 1923.*)

In a previous paper [Elliot, Crichton and Orr, 1922], the importance of the inorganic constituents of food in nutritional disorders, with especial reference to rickets in pigs, was pointed out. In this paper, attention will be drawn to the important place that another inorganic constituent, namely iron, may take in the growth and development of the same animals.

In a large breeding establishment for pigs, it was the custom to keep the sows out in the open, on pasture, till within a fortnight of their farrowing date. They were then brought into the house and put in pens with cement floors, walls etc. Their food, when in the house, consisted of fishmeal, bruised maize, and "draff" or brewers' offal in certain proportions, together with an abundant supply of water.

This diet contained very little iron. The fishmeal was of the white variety, prepared from the bones and adherent flesh of white fish, without the blood, intestines or other organs and contained only a trace of iron. (Albu-Neuberg [1906] found only a trace of iron in anchovies.) Maize contains 0·008 % and barley, from which "draff" is obtained, 0·024 % iron [Forbes, 1909, p. 152].

The sows farrowed in due course and the pigs appeared to do well till they were from three to four weeks old. Then the following train of symptoms began to appear. The pigs became dull and listless, their skins became intensely white, they developed a hairy appearance, and they took on a "stocky" or thick-set look (due to oedema of the skin). In addition to these, the breathing became pumping in character and "thumps" or spasmodic jerking of the diaphragm developed. Sudden death became a common event, until whole litters perished one after the other. Cases would be seen standing up alive and, in a few minutes, they would be seen in respiratory convulsions on the floor.

On *post mortem* examination of cases at this stage, the following appearances would be found. The animals were in an apparently very fat condition, subcutaneous fat being specially abundant. The heart would be found so dilated as actually to fill almost the entire chest cavity, the lungs being crushed back and collapsed until they were confined in the angle between the bodies of the vertebrae and the ribs. There was great effusion into the pericardial cavity, as also into the pleural cavity, and the lungs were oedematous. There was great effusion of fluid also into the peritoneal cavity, and

this fluid contained large coagulated flakes. The liver was pale in colour and showed, all over its surface and substance, minute paler areas of the size of a millet seed. These areas were in the centre of the lobules. The pancreas was extremely white in colour, resembling a piece of chalk. The kidneys were also pale, and the spleen in most cases was slightly enlarged.

The blood and tissues were sterile. The blood was extremely watery and pale, the haemoglobin in many cases being about 15 %, and the red blood corpuscles about 3,000,000 per mm^3.

Microscopic examination of the tissues showed extreme fatty change in the centre of the lobules of the liver. There was also advanced fatty degeneration of the heart muscle and of the epithelium of the secreting tubules of the kidneys. (The urine contained a very slight trace of albumin.)

In the cases which continued to live, and these were few if the disease was at all advanced and if treatment was not adopted, the following appearances developed. The pig became emaciated and ceased to grow; it became very hairy and the skin assumed a cinnamon tint and became very dry and dirty. The animal became very apathetic and no longer desired food. Many of them died subsequently, and on *post mortem* examination of such cases the following appearances were found. Owing to the resorption of the effused fluid in various parts of the body, there were adhesions between the heart and the pericardium, the pericardium and the lungs and the chest wall. The lungs were collapsed and bound down with adhesions to the angle between the bodies of the vertebrae and the ribs. Often there was a pneumonic condition in their dependent margins. The lungs were also adherent to the upper surface of the diaphragm. In the abdomen the diaphragm was fixed by adhesions to the upper surface of the liver, the various lobes of the liver being also adherent to one another and to the coils of the intestine. These latter also were attached to the spleen and to one another. Especially was this seen in the large intestine, where the coils were gummed together leaving practically no furrow between them.

The condition of matters, just described, added to the mortality caused by an epidemic of distemper in the herd on account of the lung complications developed. It, alone or in conjunction with the distemper, gave rise to an outbreak of abscesses in the face, due to caries of the temporary teeth.

The following would appear to be the explanation of the train of events just described. While the sows were out at grass, they obtained abundant iron from the grass and the soil. When, however, they were taken into the pens a fortnight before farrowing, owing to the cement floors and the nature of the food, the supply of iron ceased. In spite of this, presumably owing to a certain amount of iron stored up, they were able to carry on for a period, but, after a time, this supply was insufficient for themselves and the requirements of the growing litters of pigs. The result was that the symptoms and effects of iron deficiency as described appeared in the pigs, and the sows themselves became very emaciated. In this latter connection, it may be

mentioned that the pigs of those sows which contrived in some way to keep up their condition suffered relatively less from the disease.

The milk of swine contains 0·009 % of iron as compared with 0·002 % in the cow, thus showing a high demand in the pig [König, 1920]. There is a storing up of iron in the body of the unborn mammal in anticipation of the poverty of milk in this essential nutrient. During the whole of the suckling period, the young animal is drawing upon this reserve and lowering the percentage in its body [Bunge, 1889]. Hess, Unger, and Supplee [1921], show that the iron content of milk varies with the feed of the animal. Thus cow's milk contains twice as much iron when the animals are fed on pasture as when they are fed on a mixture of bean meal, linseed meal, hominy, gluten meal, and bran together with dried beet pulp, molasses and straw (an anti-scorbutic-free ration).

At the time when it became evident that deficiency of iron was the cause of the disease, there were in the piggery two groups of pigs, first, those over three weeks old, already in the grip of the disease with the organic changes in the liver, heart, kidneys and lungs, and second, those under three weeks old down to those newly born, in which the disease had not begun or where at least it was not so advanced. The mothers of both sets, as well as those pigs which were already feeding for themselves, were at once put on to large doses of ferric oxide, which was administered in their food. The effect of this was noticeable almost at once in several directions. The sudden deaths ceased at once. The pigs became more lively and had more appetite for their food, those of them, which had arrived at the stage of feeding for themselves, now squealing whenever they heard a bucket rattled. Examination of the blood of about a hundred of them, before and after the administration of the iron, showed that the haemoglobin had risen in about three weeks from 20–30 % to 70–80 %. This return of the haemoglobin to about normal did not, and could not be expected to, remove the disease symptoms entirely, owing to the organic changes in the liver, lungs and elsewhere which its lack had produced. No amelioration could for instance be expected in the case of the collapsed adherent lungs, the adherent pericardium and the adhesion of the various organs in the abdomen. The iron, however, had a marked effect in removing the fatty changes in the various organs. This was especially noticeable in the liver, which became normal in consistence and colour, except where, in some cases, the process of regeneration of the liver tissue could be seen in raised darker coloured ring-like areas—like "fairy rings"—on the surface and in the substance of the liver.

The effect of the administration of the iron to sows, before their pigs became visibly affected with the disease, may be judged of by the fact that, apart from the cessation of the sudden death condition, the pigs now were normal and healthy and the pigs of such litters at eight weeks old were about three times the size of the diseased pigs at fourteen weeks old (37 pounds as compared with 14 pounds).

The disease described here would appear to be the same as that discussed

by (amongst others) Wither and Carruth [1918] as cotton seed poisoning. The symptoms and *post mortem* appearances are alike and Wither and Carruth used iron salts to treat the disease with beneficial results. They did not however use iron salts with the idea that the condition was due to a deficiency of iron but apparently with the view that "the iron salts combine with or facilitate the oxidation of the harmful substances in the cotton-seed meal." The only other reference in the literature which we can find bearing on what may be this disease, occurs in Hutyra and Marek [1913] where a description is given of a disease called enzootic hepatitis of pigs. The disease apparently occurs when the pigs are from two to four months old and the pathological appearances, so far as they are mentioned, resemble closely those of the disease we have described. It caused great losses in Russia and during the year 1906 "the losses from this disease in Eastern Prussia were greater than those due to Swine Plague."

The disease discussed in this paper is a very important one in the pig industry. In the instance we have described it occurred not as an experimental production, but in the ordinary course of events in a pig rearing undertaking and we have found evidence of its occurrence in a considerable number of other like concerns. Its appearance depends on the nursing sows being fed on food which for various reasons does not contain enough iron to keep pace with the demands of the growing pigs. The subject matter of this investigation may have an important bearing on the pathology of chlorosis and on the pathology and aetiology of "Wet" Beri-beri in human beings. The fact that in the latter condition the symptoms and *post mortem* appearances are very similar to those described above in the pigs and that while rice bran contains 0·232 % of iron, polished rice contains only 0·003 % [Forbes, 1909, p. 152] is suggestive in this connection. The problem is being further inquired into from these directions, as also from the general stand-point and its relation to the cotton-seed poisoning so prevalent in the United States.

Orr [1922] has emphasised the importance of the mineral content of the food in connection with the etiology of rickets. He has not however specifically mentioned iron. Sollmann [1907] points out that human milk contains about three times as much iron as the artificial foods commonly fed to infants. It is probable that deficiency of iron may be a factor in conditions of malnutrition in infants as well as calcium and phosphorus.

REFERENCES.

Albu-Neuberg (1906). Mineralstoffwechsel, S. 241.
Bunge (1889). *Z. physiol. Chem.* **13**, 399.
Elliot, Crichton and Orr (1922). *J. Exp. Path.* **3**, 10.
Forbes (1909). *Bull. Ohio Agric. Exp. Station*, No. 201, p. 152.
Hess, Unger and Supplee (1921). *J. Biol. Chem.* **45**, 234.
Hutyra and Marek (1913). Special Pathology and Therapeutics of the diseases of Domestic Animals (translated by Mohler and Eichorn), **2**, 521.
König (1920). Chemie der Nahrungs- und Genuss-mittel, etc., **2**, 871.
Orr (1922). *Lancet*, ii, 394.
Sollmann (1907). Textbook of Pharmacology, 626.
Wither and Carruth (1918). *Experimental Station Records*, **38**, 685.

XXVI. THE TECHNIQUE OF BREEDING RATS FOR FEEDING EXPERIMENTS.

By GLADYS ANNIE HARTWELL, ELSIE CHARLOTTE MOTTRAM and VERNON HENRY MOTTRAM.

(*Received February 19th, 1923.*)

Introduction. The importance of good stock for experimental purposes cannot be exaggerated. That control material should be identical with that used for experiment is axiomatic. The elimination of variations due to disease or nutrition is of such value to the experimenter on growth problems—so fundamental to his work—that we venture to publish the means we have found the best for producing good material. The work was begun by one of us (E. C. M.) in the Biochemical Laboratory in Cambridge; carried on by another (V. H. M.) in the Animal Nutrition Department at Lever Bros. and the experience gained[1], grafted on that of the third (G. A. H.) and brought to its present development by her at King's College for Women (Social and Household Science Department). Our observations and experiments have extended over more than six years.

That the present technique produces large animals is seen from the curves of growth of the animals compared with Donaldson's curves (Figs. 1 and 2). This size is not due to fat alone for it starts from birth. Nor does it interfere with reproduction, for the animals are prolific and have large sized litters. Only in the winter months does the number per litter fall below six and that rarely. In the height of the two breeding seasons it is often over twelve. Yet the mother rat is able to bring up all the litter with a high average weight (*e.g.* with 9 in litter the average weight at the 21st day is 27·6 g.; 11 in litter, 28 g.; 12 and 14 in litter, 24 and 25 g. Donaldson gives 21 g.). The animals are almost free from intercurrent disease—a fact readily acknowledged by those who take our surplus animals. Most important is that the growth curves for any one time in the year are nearly constant. The animals show the marked vivacity, the prompt reaction to auditory stimulus and the glossy thick coats of healthy rats.

Stock. The original plan[2] adopted was to buy mixed stock from the dealers, keep it under observation for some time and then to breed from the does, using large and powerful bucks. This we now consider unwise. The mixed

[1] Based as often as not on the results of mistakes as on expectations justified.

[2] Reliance on which together with too great an eagerness to get on with nutrition research before the stock was stabilised produced effects in the animal house at Spital which occupied much of the time of the successor of one of us in eliminating.

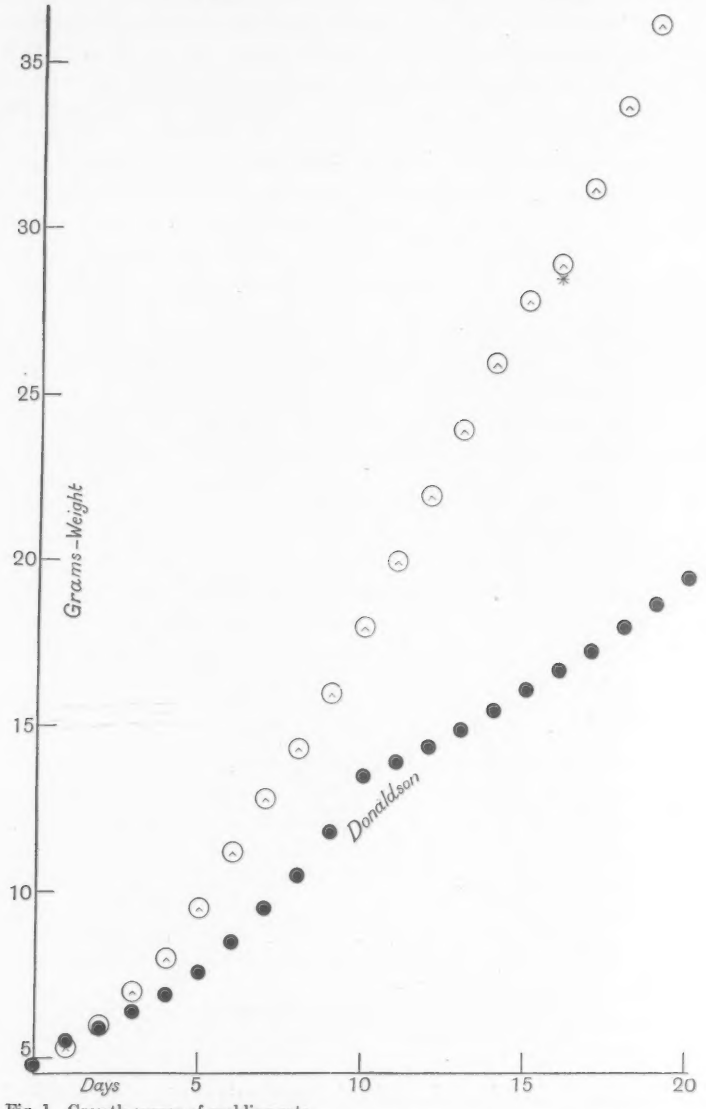


Fig. 1. Growth curves of suckling rats.
Open circles represent daily weight of rats bred by methods given in this paper.
Closed circles represent daily weight of rats as given by Donaldson.
* Animals began eating for themselves.

stock from dealers is often infected with disease which does not show up early in life and is almost impossible to eradicate. Long experience in our present laboratory (K. C. W.) backed by the similar experience of Dr C. E. Dukes, to whom we owe some of our stock, convinces us that the best way to proceed, is to begin by breeding from one or more pairs of adult animals, which have been obtained from a good source (*e.g.* another researcher) when quite young and kept on a good diet under strict observation. *Further animals should be obtained by inbreeding.* There is no evidence that inbreeding is of any danger

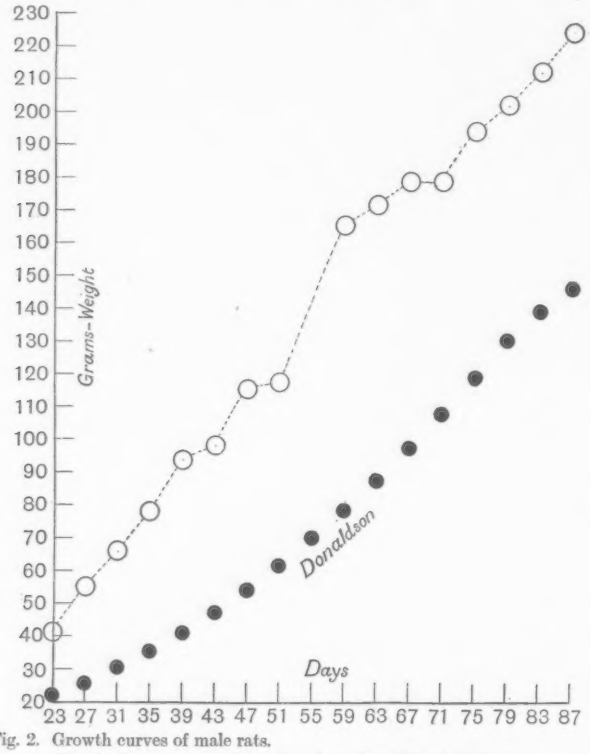


Fig. 2. Growth curves of male rats.
Closed circles are weights taken from Donaldson's book.
Open circles are weights of rats bred by methods given in this paper.

when the original stock is good. In fact inbreeding is of advantage in stabilising the stock and cutting down variations. We should be diffident in putting forward the value of inbreeding so prominently, were it not for the experience of the Wistar Institute lasting over several years, which confirms us in our impressions.

Coat colours. For preference we use black and white piebald rats as being most certain in temper and best in rearing young. Pure albinos rank next. They are less certain in temper and are not so good in rearing their first litter. When they are good mothers they are very good indeed. Other coloured rats are much more uncertain, the greys most of all. With black and white piebald rats brought up from birth or weaning by hand, so as to be used to handling, it is very rarely that one gets bitten. Whites sometimes bite when they have a litter, but much depends on how they have been handled in the past. Irish *a* and *b* rats frequently bite and the grey-piebald and self coloured have to be handled with care.

Coat colours influence the gross metabolism of the animal. In hot humid weather the whites do better than piebald black and white, and these, in turn, better than Irish *aa* and *bb* and self blacks. Such weather affects the growth and the tempers of rats very adversely. Fortunately it is not common in England.

In choice of coat colours it is well to eschew greys, self colours, Irish animals and fancy colourings and to depend on black and white piebalds or on albinos. The black and white piebalds are easier to handle and are more certain; the albinos stand heat, especially humid heat, better.

It is of advantage in experimentation to have animals variously marked. For instance in feeding experiments it is common to place pairs of the same sex in a cage together, for they like company. To know them apart it is convenient to have one albino and one piebald together, otherwise we must distinguish them by some artificial mark—ear piercing, tail clipping or what not. One of us used a simple Mendelian trick for getting equal numbers of piebald and albino rats. "Impure" piebald rats are crossed with albinos, with the result that 50 % of the offspring are albinos and 50 % piebald.

Housing. Probably most of the failure in rat breeding is due to housing. The first thing of importance is *constancy of temperature of the surroundings.* We try to keep the mean temperature 65° F. with no greater variation on either side than 5° F. Consequently we prevent, if possible, the temperature from rising above 70° F. or falling below 60° F. The animals will not breed at a low temperature. High temperature affects them adversely. Disease, especially pneumonia, is common if the temperature be not regulated. A north lighted room without great window space enables the temperature to be kept more constant. American paraffin oil stoves have been used to supplement the radiators at night with no observable detriment to the rats. Whether the duller light of a north-lighted room is an advantage or not we are unable to say. Many consider that the rat when normally fed does better in the dark than in the light. The problem of caging the animals is not difficult. Undoubtedly the animals prefer wood. It is warmer and more comfortable. But it has drawbacks in that it is more difficult to keep clean and it harbours parasites. Moreover rats are destructive and often gnaw wooden cages to pieces. Parasites, however, particularly that of scab, are easily dealt with as

described below. Some people believe that rats cannot live in metal cages. This is not true. One of us has reared generation after generation from birth to adult life in metal cages.

The present practice (G. A. H.) is to keep the pregnant and lactating does in separate individual wooden cages, $9'' \times 9'' \times 9''$, till it is time (21 days after birth) for weaning the young. Adult animals are kept in metal cages $2'\,0'' \times 1'\,6'' \times 1'\,6''$. Not more than twenty are allowed in each cage, with three or four bucks to twelve or sixteen does. The growing animals are placed in proportionately smaller metal cages of smaller mesh. For the very young, square, and not oblong, wire meshing is used for the rats can climb on it and take exercise. A photograph of the latest design, with a platform to serve as "bedroom" is given (Fig. 3). For metabolism experiments the old round metal rat cage, opening at the side, has been discarded, for a square one on legs opening from above.

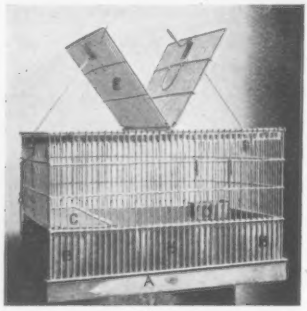


Fig. 3. Cage for adult rats $2' \times 1\frac{1}{2}' \times 1\frac{1}{2}'$. A. Tray in which the cage stands and forming its bottom. B, B. Palisade to prevent scattering of food and sawdust. C. Platform or "bedroom" on which the rats sleep. D. Drinking trough. E. Roof of cage, both sides shown open. The cage was made to our design by Messrs Baird and Tatlock (London), Ltd.

Disease. Some diseases among rats are regarded as inevitable by most researchers. Scab is an example. This disease is due to a parasite which burrows under the skin of the ears and tail. It may, unfortunately, be transmitted from animal to animal via wood. Infected shavings, wood wool or wooden cages hand on the disease. On this account one of us (V. H. M.) completely gave up the use of wooden cages and burned any shavings, etc. which had come in contact with infected animals. But we have found that wooden cages can be used and yet the disease stamped out.

The disease is easy to cure. The ears and tail of an infected animal are carefully anointed with the following mixture:

Spirit of tar	$1\frac{1}{2}$ parts
Olive oil	8 ,,
Paraffin oil	1 part

Add flowers of sulphur till the ointment is thick.

Instead of spirit of tar, creosote, in much smaller amounts, may be used and the other constituents may be varied in proportion with little harm.

Two days of application of this ointment should eliminate the disease from the animals. From the wooden cages we have got rid of the parasite by painting them within and without with a thin alcoholic solution of tar. Plenty of time was given for the alcohol to soak into the wood. Cages, disinfected thus, never harbour the parasite. In spite of severe infection prior to September 1920 when the trouble was first seriously tackled, no scab is

ever now seen, nor has it in the past ever recrudesced. Approximately 600 litters have been reared in wooden cages and no case of scab has been seen. *Scab is easily avoidable; in a well run animal house it should never occur.*

Mange, when it has occurred, has been treated with the same mixture successfully. Stock from animal dealers is likely to be mangy as well as scabby. Cure has been effected in a few days by the use of the ointment and the disease has not recurred. A loss of hair, which might be taken for mange, but is connected with diet, has been observed by one of us (G. A. H.) and will be reported on later. The common parasite, the louse, seen on elderly and sick animals, is best treated with flowers of sulphur.

An infection of the urogenital tract in does has been seen by two of us in the past in animals bought direct from a dealer. It causes a great deal of trouble. Attempts on our part, and on the part of the successor of one of us (Mr A. D. Stammers), to stamp it out, were unsuccessful. As none of the original stock, from which our present animals have been bred, suffered in that way, the disease is never seen. We should advise that any animals of unknown origin imported into an animal house, should be kept in pairs in quarantine until they produce litters, and that the litters be quarantined till they reach puberty.

Internal ear disease is sometimes seen. The animal holds its head on one side and later often starts circus movements. Such trouble seems irremediable. No correlation of it with inbreeding has been observed. It used to occur when the utmost pains were taken to secure cross-breeding. It occurs no more frequently now that the animals are intensively inbred. About twenty cases have occurred, in the last two years, in a colony which has never fallen below 300, and in summer is much larger.

Pneumonia is frequently seen. In fact it is the great danger in the rat house. The main factors affecting its incidence are temperature of the surroundings and diet. Since the surroundings of our animals have been made more equable in temperature and free from draughts; since the animals have had good food, pneumonia has greatly decreased. Outbreaks are usually referable to some unforeseen drop in temperature or a period when the "kitchen scraps" upon which the stock are fed contained too little protein.

The fact that diet causes a very appreciable difference in the frequency of the disease, makes it almost inevitable that during long-continued experiments on deficient diets, pneumonia will occur. Consequently, when possible, animals undergoing a long feeding experiment should be housed in quarters different from those of the stock. (This problem has not arisen in our present animal room, in any acute form, as the majority of the experiments have lasted three weeks only, and the diet has been adequate for every purpose except lactation.)

On the whole it does not appear to us worth while to quarantine any sick animal and try to nurse it back to health. Some vigorous, well-grown animals may recover from an incipient catarrh when quarantined and put on a good

diet. But usually the experiment does not succeed. The animal may recover temporarily, but usually succumbs again when returned to the stock cages. (This opinion must not, of course, be taken to apply to scab which can so easily and permanently be stamped out.)

Breeding. The does reach puberty at about the 90th day. On special diet they may reach it earlier. But it is not considered wise to allow impregnation till about the 6th month, when they have attained their adult stature. The present plan is then to place 16 females in a large cage with 4 well grown males. Other signs besides an increase in the size of the abdomen may help the inexperienced to judge of pregnancy, such as rapid increase in weight, uncertainty of temper, squeaking on handling, tetanic movements of fore-feet, and nest building of chance chips and straws. Any of the signs, but not all, may fail. The last two occur very shortly before the birth of the young. Experience is the best guide in judging the stage of pregnancy.

The pregnant does are removed to separate quarters, wooden boxes $9'' \times 9'' \times 9''$, ventilated in front with perforated zinc and opening with a wooden lid from above, when pregnancy is well advanced. If the does have been used to being handled from early days (birth or weaning) by a person whom they get to know, there is no difficulty in that person's handling them when they are pregnant or after the birth of the litter. Nor should the handling of the litter result in loss. Ordinarily, mothers are apt to kill and eat their young if disturbed within ten days after parturition. But when they are used to being handled by any one person from weaning, they do not resent that person's handling them or their young. Experiments which one of us (G. A. H.) has been conducting now for some years, have necessitated the daily weighing of mothers and litter from birth. Over 600 litters have been handled and it has been rare indeed to have a casualty. The main breeding seasons are spring and autumn.

The young are usually eating for themselves by the 18th day and can readily be weaned on the 21st day. The best all-round diet for the mother while the young are dependent on her, is bread and milk. No such curves of growth and no such general fitness have been seen on other diets except bread, "food casein" and egg yolk; bread, "food casein" and whey; and bread, "food casein" and excess marmite.

After weaning, the general practice is to put twenty youngsters, with perhaps one adult male as foster parent, together in one of the cages with smaller squared mesh, promoting them to larger cages as they grow. The sexes are separated early. The diet given is bread and milk supplemented with kitchen scraps. Care has to be taken with the kitchen scraps lest they contain too high a percentage of vegetable material and not enough protein and fat. The best kitchen refuse for feeding purposes contains meat, milk puddings and fish. If it is noticed that the quality of the kitchen scraps is poor, it is usual to supplement it with fresh herrings or some cheap source of animal protein and fat on the market. Animals fed on this diet grow large

and healthy and produce large litters. Adult does under 200 g. rarely occur (when they do, they are used for histological purposes) and animals of 300 g. are frequent. The males sometimes weigh 400 g.

A final point is that personal supervision every day by the researcher who is using the animals is almost essential. However good paid assistants are, it has been our repeated experience that animals do better when the person in control of the experimental work sees them and works in the animal room every day. Daily supervision, with an inspection of every animal in the animal and experimental rooms at least twice a week, should be the aim of anyone who wants good animal material upon which to work.

Summarising our experience we would say that good rat stock may be best obtained by

(i) housing at an equable temperature of 65° F. with no more than 5° variation in either direction;

(ii) feeding on diets containing bread and milk and kitchen scraps with a high percentage of protein and fat;

(iii) inbreeding from originally good stock using does of six months old;

(iv) feeding the mothers during lactation with bread and milk;

(v) inspection and handling of stock from birth.

Thanks are due to the Medical Research Council for grants defraying the cost of the majority of the experiments in preparation for which the above experience has been gained and to Messrs Lever Bros. Ltd. for permission to publish anything bearing on work done for them.

XXVII. OXIDISING ENZYMES. VI. A NOTE ON TYROSINASE.

By MURIEL WHELDALE ONSLOW.

(*Received February 26th, 1923.*)

In several previous communications [Onslow, M. W., 1920, 1921], evidence has been given in support of the hypothesis that many plants contain enzymes, termed oxygenases, which catalyse the autoxidation of aromatic compounds having two hydroxyl groups in the ortho position, with the formation of peroxides: the latter are then acted upon by peroxidases and produce "active" oxygen. The complete system, *i.e.* oxygenase, aromatic compound and peroxidase, constitutes an oxidase (phenolase or laccase).

There is another oxidising enzyme, termed tyrosinase, which has been recognised in both the animal and vegetable kingdoms. Two characteristic reactions of tyrosinase are the oxidation of tyrosine with the production, finally, of a black pigment (melanin), and the oxidation of *p*-cresol with the formation of a deep orange colour.

In regard to tyrosinase, Bach [1914] holds the view that it consists of two enzymes: a deaminase which brings about the deaminisation of the amino group of tyrosine with the production of *p*-hydroxyphenylacetaldehyde, carbon dioxide and ammonia, and an oxidase (phenolase) which oxidises the molecule giving the black pigment (melanin).

The purpose of the present note is to introduce the following new observations and suggestions. After summary statement, they will be considered in more detail.

1. All plants examined which give the tyrosinase reaction also contain oxidase.

2. It is suggested (in corroboration and extension of Bach's hypothesis) that tyrosinase is a mixture of enzymes, *i.e.* a water-splitting enzyme (reductase or deaminase), a carboxylase and an oxidase (consisting of oxygenase, aromatic substance and peroxidase).

3. In order to account for the action, on tyrosine, of the tyrosinase system from which the aromatic component of the oxidase has been removed by washing with alcohol, it is suggested that tyrosine itself may supply a substrate in the following way. Traces of dihydroxyphenylalanine may be formed from tyrosine in the presence of traces of "active" oxygen. Dihydroxyphenylalanine would then act as a substrate for the oxygenase, constituting, in conjunction with peroxidase, a system for producing "active" oxygen, and the reaction would be continuous. "Active" oxygen is also accepted by the

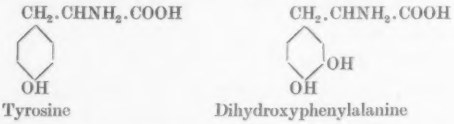
hydrogen set free by the reductase, and hence the products of reaction do not blue guaiacum.

4. The *p*-cresol reaction in plants appears to be due to oxidase alone. It is suggested that an additional hydroxyl group is introduced, as in the case of tyrosine, giving rise to an *ortho*-dihydroxy-compound which provides a substrate for the activity of the oxygenase. In the presence of peroxidase, "active" oxygen is formed and accumulates, and hence the products of the reaction blue guaiacum.

5. The instability of oxygenase is a cause of loss of activity of tyrosinase. The activity can be restored to a certain extent by hydrogen peroxide.

Now, to consider some of the above points in more detail. Some 21 plants (representing 15 Natural Orders) examined have been found to contain tyrosinase. All, in addition, contained the oxidase system previously described [Onslow, M. W., 1920]. In fact, no tyrosinase plant has yet been found which does not contain oxidase, and it appears probable that oxidase is a constant component of tyrosinase in the plant. The enzyme extract used for demonstrating the presence of tyrosinase was made exactly as for oxidase, *i.e.* by quickly and thoroughly pounding the tissues with 98 % alcohol and repeatedly filtering. The residue, extracted with water, gives the enzyme extract.

This extract, though practically free from the aromatic component of the oxidase, yet slowly blackens tyrosine. If an oxidase is essential to the action of tyrosinase, it would appear that, though the aromatic constituent is absent, the oxidase still functions in this case. Since it is almost impossible to obtain an enzyme preparation as above which does not give a slight blue colour with guaiacum tincture, a trace of "active" oxygen is consequently always present in the extract. The suggestion is, therefore, that in the presence of these traces of active oxygen, a second hydroxyl group is introduced into tyrosine with the resultant formation of dihydroxyphenylalanine:



Tyrosine Dihydroxyphenylalanine

Even if such a compound were only present in traces, it would immediately act as the substrate for the oxygenase, and supplies of peroxide and hence "active" oxygen would be produced; that is, the reaction would be continuous. Further, in confirmation of Bach's view, the "active" oxygen thus produced may also act as an acceptor for the hydrogen produced by the reductase: thus the first process might be represented:

$$\underset{\text{tyrosine}}{C_6H_4(OH).CH_2.CH(NH_2).COOH} + O \downarrow \underset{\text{water}}{H_2}$$
$$\rightarrow C_6H_4(OH).CH_2.COH(NH_2).COOH$$
$$\rightarrow C_6H_4(OH).CH_2.CO.COOH + NH_3$$
$$\rightarrow C_6H_4(OH).CH_2.CHO + CO_2[1]$$

[1] Probably catalysed by carboxylase.

and the second process:

Tyrosine + traces of "active" oxygen → dihydroxyphenylalanine
Dihydroxyphenylalanine + oxygenase → peroxide + other oxidation products
Peroxide + peroxidase → "active" oxygen

"Active" oxygen should not accumulate when the whole reaction takes place, and this is borne out by the fact that the products of the action of tyrosinase on tyrosine do not blue guaiacum. The complete process of oxidation of the tyrosine molecule involving melanin formation is doubtless complex, since the ammonia and the aldehyde are eventually involved.

Further evidence in support of the above suggestion is the statement, made by Dakin [1922], that hydrogen peroxide is able to oxidise monophenols to diphenols.

Bach has been unable to prepare tyrosinase free from oxidase: if free from oxidase, hydrogen peroxide is necessary and must be added to obtain a positive reaction (melanin formation). Peroxidase is always essential.

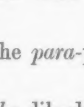
In this connexion Bach, moreover, has shown that very dilute hydrogen peroxide much increases the activity of tyrosinase itself, and may, as mentioned above, replace to a certain extent, the oxygenase-peroxide component of tyrosinase. Thus, it would assist the activity of the oxygenase, if this is present, by forming dihydroxyphenylalanine, or it might, as Bach suggests, supply active oxygen for the whole reaction for a time.

Unlike Bach, Chodat [Chodat and Wyss, 1922] maintains that he has prepared tyrosinase free from oxidase (laccase, phenolase) but, as he tested with guaiacum, he might quite well have no, or very little, reaction since his enzyme, on account of the mode of preparation, would contain only oxygenase and peroxidase and no substrate for the former. He also claims to have a preparation of tyrosinase, from one source, free from peroxidase but does not definitely state how many tests he employed to detect peroxidase.

Dihydroxyphenylalanine, itself, occurs in the vegetable kingdom, being found, for instance, throughout the tissues of the Broad Bean (*Vicia Faba*) [Guggenheim, 1913]. The plant, on injury, turns jet black owing to the oxidation of this substance. Dihydroxyphenylalanine has also been suggested as a chromogen which gives rise, through the activity of tyrosinase, to melanin pigment in animals. A solution of the crystalline product prepared from *Vicia* rapidly turns red and then black when acted upon by tyrosinase, but the products do not blue guaiacum.

The activity of the oxygenase is an important element in the whole process of tyrosinase action. If the oxygenase is impaired, as it is very readily by alcohol, heat, etc., the tyrosinase reaction is either weakened or disappears entirely, since there is no mechanism for bringing about the constant supply of "active" oxygen essential for the complete continuance of the reaction. Moreover, oxygenase is separated from peroxidase, more or less, by fractional precipitation with alcohol [Onslow, M. W., 1920], and this may well explain the deterioration of tyrosinase preparations on reprecipitation by alcohol and

the failure to purify the enzyme by this method. The oxygenase of the Basidiomycetes is said to be more resistant; hence tyrosinase from this source is more readily prepared [Bach, 1914].

Now, to turn to the *p*-cresol reaction. This substance, as in the case of tyrosine, has one hydroxyl group in the *para*-position,  , and might, in the same way, be converted into an *ortho*-dihydroxy-derivative by "active" oxygen; or traces of a dihydroxy-derivative may even be present in a solution of the substance itself. The *p*-cresol reaction has been found to be given not only by extracts (prepared as above) from tyrosinase plants, but also by extracts from all oxidase plants examined. Thus it appears to be due to oxidase, and traces of a dihydroxy-derivative from *p*-cresol may act as a substrate for the oxygenase. In the case of *p*-cresol, however, there is no reducing action, as there is no amino group. Hence "active" oxygen should accumulate, and this is so, since the products of the reaction blue guaiacum.

Apparently it is only with substances having the hydroxyl in the *para*-position that this suggested phenomenon of oxidation to a diphenol can take place. No other phenol, except catechol, as shown in earlier papers [Onslow, M. W., 1920], can act as a substrate for the oxygenase in the same way.

(Note added April 9th, 1923.) Professor Chodat has very kindly sent me a sample of tyrosinase from *Russula*, giving, on his authority, a slight reaction for peroxidase (guaiacum and hydrogen peroxide). This I confirmed: the sample gave, in addition, a powerful oxygenase reaction (catechol and guaiacum).

In conclusion, my thanks are due to Dr F. F. Blackman, F.R.S. for kindly making criticisms and suggestions.

REFERENCES.

Bach (1914). *Biochem. Z.* **60**, 221.
Chodat and Wyss (1922). *Compt. Rend. Soc. Phys. et Nat.* Genève, **39**, 22.
Dakin (1922). Oxidations and Reductions in the Animal Body.
Guggenheim (1913). *Z. physiol. Chem.* **88**, 276.
Onslow, M. W. (1920). *Biochem. J.* **14**, 535.
—— (1921). *Biochem. J.* **15**, 107.

XXVIII. ÉTUDE DE L'ALIMENTATION ARTIFICIELLE CHEZ LE PIGEON ET DE LA DÉFICIENCE EN VITAMINES.

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Directeur: Mr le Professeur F. MALENGREAU.

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LES essais d'alimentation artificielle des pigeons que nous poursuivons depuis plus d'un an dans le but d'étudier les déficiences en vitamines, nous permettent de compléter aujourd'hui les données de notre premier mémoire sur ce sujet [Hoet, 1922].

Le régime de nos animaux, resté presque identique à celui dont nous avons décrit la préparation, est composé de: caséïne 18, amidon de riz 60, mélange de sels de McCollum 4, papier filtre 3, margarine 10, beurre 5 parties.

Ce régime de fond est déficient en vitamine B. Pour le rendre complet et parer à cette déficience, on y ajoute de la levure ou toute autre substance riche en facteurs hydrosolubles.

Caséïne et amidon. La caséïne et l'amidon employés sont ceux livrés par le commerce. Ces produits, comme l'a constaté Osborne, comme nous l'avons vérifié nous-même par des expériences sur des rats, sont pratiquement dépourvus de vitamine B. Nous nous en sommes tenu à la proportion de 18 % de caséïne, largement suffisante pour couvrir les besoins azotés de tous nos animaux, jeunes et adultes. Mais nous avons pu, chez ces derniers, abaisser la proportion à 12 %, pendant plus de trois mois, sans qu'il en résulte aucun trouble de nutrition. Nos résultats sur les pigeons cadrent ainsi avec ceux qu'Osborne a lui-même obtenus dans ses expériences sur les rats [Osborne, 1916].

Le beurre est soigneusement lavé plusieurs fois à l'eau tiède pour en écarter toutes les substances solubles. Le papier filtre est le papier ordinaire du laboratoire.

Levure. La levure qui a servi à combler la déficience en vitamine B, est la levure séchée du commerce, qui contient encore approximativement 10 % d'humidité.

On peut l'employer comme telle, délayée dans l'eau, mais il est préférable d'en faire l'extrait aqueux. On écarte par là les protéines coagulables et on facilite l'absorption des facteurs accessoires. Nous avons, en effet, observé

que la quantité de levure nécessaire pour maintenir l'équilibre du pigeon est plus grande avec la levure entière qu'avec le produit de sa décoction. Cela tient vraisemblablement à une digestion difficile et incomplète des cellules de levure dont une partie passe dans les fèces, comme Funk [1916] l'a observé chez l'homme.

L'extrait aqueux que nous avons couramment employé est préparé de manière à permettre des mesures comparatives de son activité: 200 g. de levure sèche délayés dans deux litres d'eau environ, sont chauffés rapidement et maintenus à une vive ébullition pendant cinq minutes. Après refroidissement, on porte la masse totale à deux litres et on la jette sur un ou plusieurs filtres. On obtient un filtrat limpide, plus ou moins coloré, dont chaque centimètre cube contient ainsi l'extrait de 0·1 g. de levure. Le filtrat est évaporé au bain marie jusqu'à une concentration cinq ou dix fois plus grande. Cette méthode, courante en chimie analytique, évite les lavages toujours ennuyeux et permet d'obtenir une solution d'activité constante; elle est applicable ici, le résidu insoluble n'ayant aucun pouvoir adsorbant vis-à-vis des facteurs accessoires. On peut concentrer l'extrait davantage encore et lui enlever une partie de ses sels en les précipitant par addition d'un volume égal d'alcool à 95° qu'on chasse, après filtration, par distillation sous pression réduite.

Au lieu d'incorporer l'extrait de levure à la pâte alimentaire comme on le fait généralement pour les rats, nous préferons le donner séparément à intervalles réguliers de un, deux ou trois jours. La quantité que reçoit le pigeon, est ainsi exactement mesurée et on évite les influences psychiques que peut avoir sur l'appétit de l'animal toute modification de saveur ou d'odeur de la nourriture.

Influence des vitamines B sur l'appétit des pigeons.

Nous avons montré dans notre premier mémoire comment le régime rendu déficient par suppression de la levure, détermine aussitôt chez le pigeon une diminution d'appétit avec perte progressive de poids aboutissant après un nombre variable de 8 à 20 jours aux manifestations convulsives de la polynévrite aviaire. Cette diminution d'appétit est de règle chez les rats où Funk l'avait signalée dès 1914 [Funk, 1915]. Mais à l'encontre de ce qui se passe chez ces derniers où l'anorexie est tardive et ne se répercute sur la courbe de poids qu'après deux à trois semaines de régime déficient, l'effet, chez le pigeon, est immédiat et peut prendre moyennant certaines précautions, une allure d'une grande régularité. De toutes ces précautions, la plus importante est d'avoir un pain toujours également frais, distribué à la même heure avec une eau journellement renouvelée. On peut mesurer l'appétit, soit par la quantité ingérée d'aliments, soit par les variations de la courbe pondérale. Dans les deux cas les chiffres obtenus présentent des variations comparables. Le tableau I indique les pesées de trois pigeons observés pendant une période de quinze jours. Tous les trois reçoivent du régime déficient *ad libitum*. On leur donne en plus tous les quatre jours le quadruple ou un peu moins de la dose

journalière minima de levure, ce qui a pour résultat de les faire manger, gloutonnement pendant la première journée, puis de moins en moins pendant les jours qui suivent; leur courbe de poids affecte ainsi l'aspect d'une ligne brisée presque régulière avec des hauts et bas coïncidant respectivement au jour le plus proche et le plus éloigné de l'ingestion de levure.

Tableau I.

Dates juillet	Poids des pigeons No. 19 g.	No. 31 g.	No. 36 g.	Quantité donnée de levure (sous forme d'extrait) g.
6	400	343	388	2·4
7	370	323	365	—
8	360	312	350	—
9	348	310	336	—
10	370	317	364	2·4
11	377	312	380	—
12	358	308	349	—
13	351	304	349	—
14	382	312	350	3·0
15	365	334	372	—
16	360	320	361	—
17	362	314	355	—
18	373	318	366	3·0
19	354	315	358	—
20	359	320	355	—
21	348	313	346	—

Sans obtenir toujours une régularité aussi grande, il est incontestable qu'un des effets immédiats de la vitamine B est de stimuler fortement l'appétit, et cela, dans des conditions où l'on peut exclure toute influence psychique pouvant provenir de la saveur particulière des aliments.

Il serait inexact cependant, et plus personne n'y songe aujourd'hui, de considérer la vitamine B comme un simple apéritif et de confondre l'avitaminose avec l'inanition; la quantité de nourriture ingérée diminue mais ne tombe jamais à zéro. Les études de Terroine et Barthélemy [1922, 1, 2] sur les manifestations comparées de l'inanition et de la déficience sont d'ailleurs décisives à cet égard.

Dose minima de levure.

La mesure de la dose minima de levure nécessaire pour prévenir les manifestations de la déficience n'est pas toujours facile. On connaît la discordance entre les chiffres mis en avant par les divers auteurs à propos de la quantité requise pour la croissance des jeunes rats. Outre les différences individuelles qu'on relève d'un animal à l'autre, il faut tenir compte de ce que les levures ont une richesse en vitamine B variable d'après la nature du moût sur lequel elles se sont développées. Tout récemment encore Kennedy et Palmer [1922] mettaient en doute la valeur de la levure comme source de vitamine B, dans les expériences de nutrition sur les rats à cause de sa grande irrégularité. Nos résultats ne sont donc valables que pour les conditions où nous avons opéré.

Chez les pigeons, d'une façon générale, quels que soient l'âge et le sexe,

les différences individuelles nous ont paru moins grandes que chez les rats. L'extrait de 1 g. de notre levure sèche a toujours été suffisant, soit pour prévenir la polynévrite avec maintien du poids normal, soit pour la guérir avec retour rapide au poids initial. La dose minima oscille habituellement entre 0·80 et 0·90 g. pour des pigeons de 350 à 400 g.; elle semble fléchir quand diminue le poids de l'animal.

Chez les pigeons de 250 à 300 g., en pleine déficience, avec phénomènes convulsifs, une dose quotidienne d'extrait de 0·60 g. de levure suffit pour supprimer en quelques heures les manifestations nerveuses et provoquer une augmentation de poids, mais le relèvement de la courbe pondérale est lent et n'atteint jamais le niveau normal.

De l'ensemble de nos recherches sur l'alimentation artificielle des pigeons, nous sommes en mesure aujourd'hui de conclure que notre mélange d'aliments purifiés, quand il est complété par de l'extrait de levure comme source de vitamine B, répond à toutes les exigences d'une alimentation complète: il maintient sans altération aucune l'équilibre physiologique de nos animaux, assure, comme nous allons le voir, la fonction de reproduction et permet la croissance des petits. Au cours de 18 mois d'essais, une soixantaine de pigeons ont été nourris exclusivement avec ce mélange, *que tous mangent spontanément*, sans que nous ayons observé un seul cas de dégout ou d'intolérance même passagère. Le tableau II donne quelques renseignements sur les pigeons actuellement en expérience et qui n'ont jamais cessé d'être au régime artificiel exclusif malgré des déficiences plusieurs fois provoquées.

Tableau II.

	Régime artificiel		Poids des pigeons			
Numéros des pigeons	Date du début	Durée actuelle (mois)	Au début g.	Au 20. ii. 23 g.	Nombre de crises de déficience	Observations
2	16. ix. 21	17	390	415	6	
3	14. x. 21	16	430	430	5	
10	10. xii. 21	14	450	420	5	
12	14. xii. 21	14	465	400	6	
22	12. xii. 21	14	520	435	4	
25	10. ii. 22	12	430	370	2	
26	10. ii. 22	12	455	465	1	
27	10. ii. 22	12	365	355	1	A pondu 6 œufs
39	16. iii. 22	11	390	392	2	
40	16. iii. 22	11	375	387	2	
50	1. iv. 22	10	290	370	1	
51	1. iv. 22	10	252	410	1	
52	1. iv. 22	10	275	420	1	Jeunes pigeons pris à l'âge de 4 à 5 semaines
53	1. iv. 22	10	280	383	1	
60	15. viii. 22	6	310	400	1	
63	15. viii. 22	6	284	430	1	

Le régime artificiel permet ainsi des études sur l'alimentation des pigeons superposables à celles qui se font sur les rats. Là comme ici, une alimentation

pour être complète, c'est-à-dire pour répondre à tous les besoins de l'organisme, devra assurer pendant une longue période l'équilibre physiologique de l'animal. La condition est formelle: une substance ne sera active comme vitamine B que si elle a une action à la fois préventive et curative autant sur la courbe de poids que sur les accidents nerveux de la polynévrite aviaire. Le poids des animaux, à la fin d'une expérience, devra donc s'être maintenu ou être revenu au niveau initial et, dans ce dernier cas, s'y maintenir encore pendant deux ou trois semaines au moins par la seule addition de la substance au régime déficient. Ces conditions ont toujours été exigées dans les recherches sur l'alimentation chez les rats, et il est étonnant qu'on en ait fait si peu de cas dans les expériences sur les pigeons.

Le fait d'empêcher la crise de se produire endéans les 50 jours, mais avec des pertes de poids allant de 20 à 30 % n'est pas un critère suffisant d'une action préventive. Autant considérer comme satisfaisante une alimentation qui, si elle était continuée plus longtemps, aboutirait finalement au dépérissement et à la mort.

Les erreurs commises dans l'étude de la vitamine B tiennent à deux causes: à l'absence de régime artificiel pour pigeons et à l'importance exagérée qu'on a trop longtemps attribuée aux phénomènes nerveux de la crise polynévritique. Ceux-ci ne sont nullement pathognomoniques de la déficience en vitamine B: ils relèvent de causes multiples et variées et cèdent à des substances qui n'ont entre elles aucun lien ni chimique ni biologique. Tout récemment, Koskowsky [1922] a montré que des injections d'histamine chez des pigeons en déficience préviennent la crise sans empêcher la baisse de poids ni la mort. La tyramine [Lipschitz, 1923] se comporte de même. Ces substances, il y a quelques années encore, auraient passé pour vitamines antinévritiques. En réalité leur pouvoir curatif sur les phénomènes convulsifs n'est pas à confondre avec l'action des facteurs accessoires.

Le régime artificiel permet l'étude comparative de l'alimentation chez le pigeon et chez le rat; c'est ainsi que du seul point de vue des facteurs hydrosolubles et en admettant l'hypothèse possible mais non encore démontrée, que la vitamine antinévritique est identique aux facteurs B de croissance, nous voyons le pigeon particulièrement sensible à cette sorte de déficience. Extrêmement tardive chez le rat adulte, n'arrêtant la croissance des petits qu'après deux ou trois semaines, la déficience a chez les pigeons au contraire un effet immédiat qui permet en quelques jours de la déceler. Aussi croyons-nous ces animaux appelés à rendre de grands services encore dans l'étude des vitamines.

Reproduction et croissance.

Une alimentation n'est réellement complète pour un animal que si elle assure la reproduction de l'espèce et permet la bonne croissance des petits. C'est à côté de beaucoup d'autres, une raison suffisante pour rejeter, en ce qui concerne les pigeons, le régime de riz poli additionné de levure [Seidell, 1922].

Nous devions nous demander si notre régime artificiel était à même de réaliser cette condition. Etant donné les résultats obtenus chez les rats avec une alimentation pareille à la nôtre, nous pouvions l'espérer. Nos essais qui se sont prolongés pendant une période de huit mois, depuis le printemps jusqu'au milieu de l'hiver 1922, ont confirmé nos prévisions. Pendant toute cette période la ponte a été régulière chez les quatre femelles soumises au régime artificiel depuis plus de quatre mois et qui, ensemble, ont pondu 27 œufs d'apparence normale avec des jaunes bien colorés.

Malheureusement les conditions expérimentales sont fort défavorables à la couvaison. De fait, nous n'avons obtenu que deux éclosions sur 20 œufs couvés. Les autres contenaient des embryons normaux morts à des périodes variables de leur développement, parfois très rapprochées du terme. Mais un seul résultat positif a ici une valeur démonstrative que n'ont pas les essais négatifs. Les deux jeunes pigeons, soumis à leur tour au régime artificiel se sont parfaitement développés (Graphique I, nº 29 et 70). Nous avons complété ces expériences en élevant, par gavage cette fois, deux autres pigeonneaux âgés de huit jours (Graphique I, H_1 et H_2), et six autres âgés de quatre à cinq semaines (Tableau II). Tous ont eu une croissance normale et sont aujourd'hui des animaux adultes de belle apparence.

Nous étions occupé à la rédaction de ces notes quand nous est parvenu le travail de Benedict et Sugiura [1923]. Ces auteurs qui n'ont pas eu connaissance de notre premier mémoire ainsi que de celui de Simonnet [1921], ont entrepris, simultanément avec nous, des essais identiques d'alimentation artificielle chez les pigeons. Leurs conclusions en ce qui concerne l'équilibre physiologique, la reproduction et la croissance de leurs animaux, sont pareilles aux nôtres et les confirment.

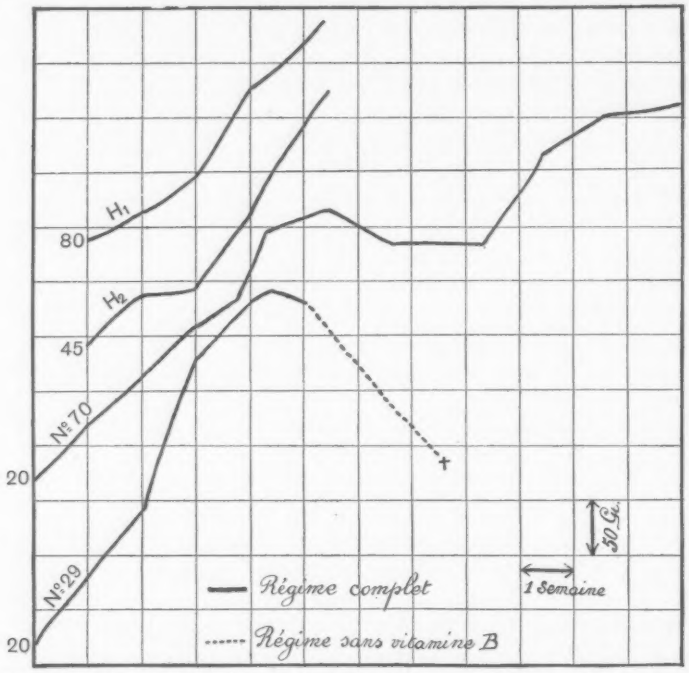
Déficience en vitamine A.

Nos expériences sur la déficience en vitamine A chez les pigeons nourris artificiellement, sont en cours depuis cinq mois. Nous aurions voulu les prolonger davantage, mais le travail de Benedict et Sugiura dont nous venons de parler et qui traite du même sujet, nous oblige à quelques remarques et à la publication, dès à présent, d'une partie de nos résultats.

L'étude des vitamines liposolubles se heurte à deux sortes de difficultés qui sont, l'une, la purification malaisée des aliments qui entrent dans la composition du régime, et l'autre, la lenteur que mettent les animaux à répondre aux déficiences en facteurs A, par opposition à ce qui se passe pour les facteurs B. Dans leur travail critique sur la question, Osborne et Mendel [1921] insistent sur ces difficultés: "...(the) removal of the fat-soluble vitamine from even purified proteins and carbohydrates is accomplished with far greater difficulty than has been hitherto suspected. An entirely convincing crucial experiment, in which nutritive failure immediately ensues upon the administration of diets fully adequate in every respect except for the presence of fat-soluble vitamine, remains to be made. It is significant that older rats thrive

for a longer time than the younger ones on the same diets, nearly, if not entirely, free from the fat-soluble vitamine. This is in contrast with the well established fact that at all periods the lack of water-soluble vitamine is speedily manifested." Ils disent également en parlant de la vitamine A: "...some, like ourselves, have described their animals as usually growing at a nearly, or quite, normal rate for periods of 60 to 80 days before showing signs of a nutritive deficiency in the diet."

Sans nier l'intérêt du travail de Benedict et Sugiura ni contredire leurs résultats, il faut reconnaître cependant que leurs essais ne sont pas à l'abri de



Graphique I. Croissance de jeunes pigeons mis au régime artificiel complet dès leur naissance. Le pigeon 29 est mort au cours d'une expérience de déficience en vitamine B.

toute critique. Une de leurs conclusions est la suivante: "Pigeons on a diet of sufficient caloric value, even though the diet lacks fat and fat-soluble vitamine, may maintain excellent condition, and may produce fertile eggs and rear healthy squabs. *Hence fat-soluble vitamine is not essential in any stage of avian nutrition.*"

Nous voulons bien admettre que les aliments qui entrent dans la composition des régimes B et C de ces auteurs, soient totalement dépourvus de vitamine A, il n'en reste pas moins que leurs expériences ont une durée qui ne dépasse pas 70 jours pour la plus longue et qui, souvent même, ne va au

delà de 30 jours quand il s'agit de la croissance des jeunes pigeons. Cette durée est manifestement insuffisante et enlève aux expériences une bonne part de leur valeur de démonstration.

Nous avons employé pour unique matière grasse l'huile d'arachide durcie par hydrogénation (hardened fat)[1], exempte de vitamine A [Committee of the Royal Society, 1919; Drummond, 1922; Report on accessory food factors, No. 38, 1919].

Notre régime avait ainsi pour composition: caséïne 18, amidon 60, huile d'arachide 15, sels 4 et papier filtre 3 parties; l'extrait de levure était donné séparément à la dose de 1 g. par jour et par pigeon. Nous avons tenu néanmoins à vérifier encore la déficience en vitamine A du mélange par un essai sur quatre jeunes rats: leur croissance s'est arrêtée après quelques semaines et leur poids a diminué progressivement. La xérophtalmie est apparue au troisième mois chez l'un d'eux, qui est mort peu après. Chez un second les manifestations oculaires se sont déclarées après trois mois et demi.

Depuis le 27 septembre 1922 ce régime est donné *ad libitum* à six pigeons (les nos 51, 52, 53, 50, 26 et 27), tous les six au régime artificiel complet depuis plus d'une demie année. L'expérience dure maintenant depuis cinq mois et rien jusqu'à présent, ni dans l'aspect général, ni dans le plumage, ni dans la température, ni dans le poids de nos animaux ne traduit le moindre trouble. Bien mieux, une femelle vient de pondre deux œufs et les couve actuellement. Nous donnons ci-dessous la courbe pondérale de ces six pigeons (Graphique II). Ces courbes présentent vers la quatrième semaine du régime déficient en facteurs A un fléchissement prononcé suivi, 15 jours après, d'un relèvement rapide, dû à une insuffisance, involontaire et momentanée de levure[2]. Cet accident que nous n'avions pas cherché, montre que les pigeons sans vitamine A se comportent vis-à-vis d'une déficience juxtaposée de vitamine B, comme des pigeons normaux.

Les expériences, bien qu'inachevées, confirment partiellement les conclusions de Benedict et Sugiura et montrent l'inutilité, au moins pour le pigeon adulte, des facteurs liposolubles.

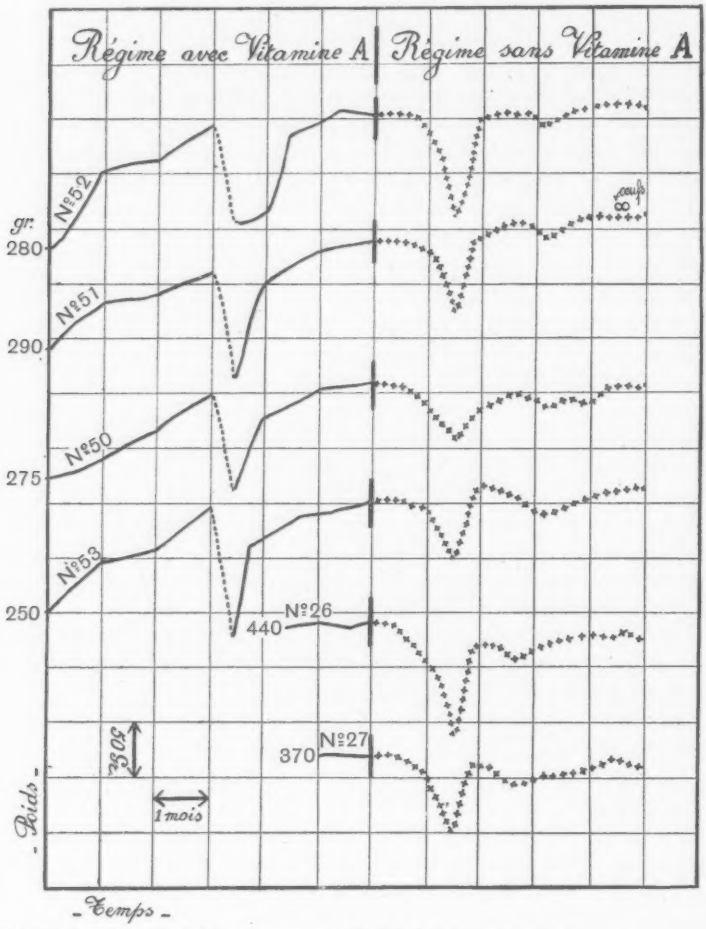
Ces résultats peuvent paraître étranges si on les compare à ceux qu'on obtient avec des poussins dans des conditions presque identiques. Hart, Halpin et Steenbock [1922] dans leur étude du "leg-weakness," signalent en effet l'importance de l'huile de foie de morue pour ceux qu'on nourrit artificiellement. Emmett et Peacock [1922] décrivent de leur côté toute une symptômatologie de l'avitaminose à chez les jeunes poules: anémie, œdème, mauvais état des plumes, début de xérophtalmie, etc.... Plimmer et Rosedale

[1] Cette huile a été mise gracieusement à notre disposition par la firme "Jurgens" de Zwyndrecht. Nous sommes heureux de leur en exprimer ici notre vive reconnaissance.

[2] Cette insuffisance était due à ce que la dose quotidienne de un gramme de levure, au lieu d'être donnée sous forme d'extrait, l'avait été pendant les six premières semaines sous forme de levure entière délayée dans la pâte. Lorsque nous eûmes substitué l'extrait à la levure comme telle, les symptômes de déficience disparurent immédiatement. Tous les autres pigeons du laboratoire ont simultanément et pour la même raison, présenté les mêmes phénomènes (voir p. 221).

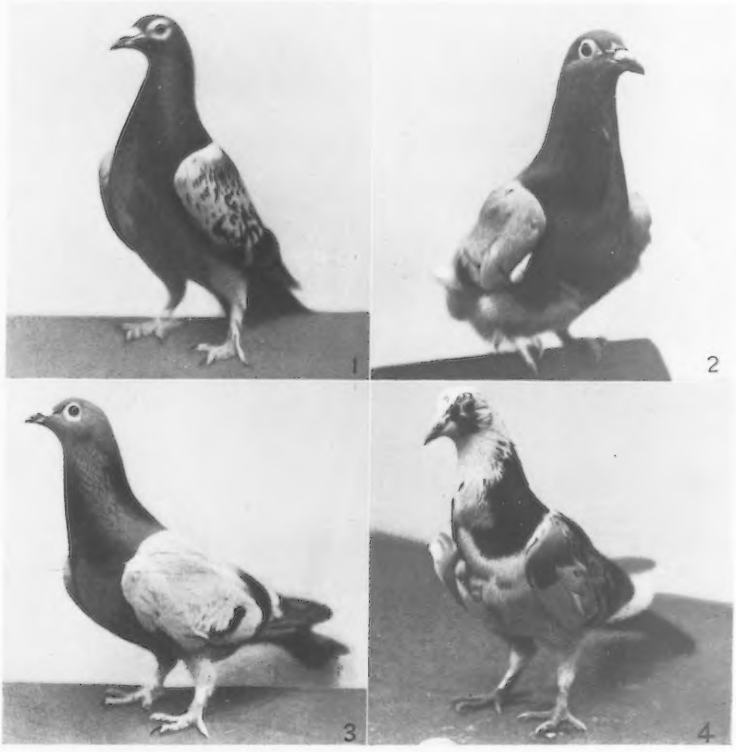
[1922] et d'autres [Nelson, Lamb and Heller, 1922] concluent, eux aussi, à la nécessité de la vitamine liposoluble dans leur alimentation.

De l'avis de tous, l'élevage des poussins est difficile et nous même l'avons tenté, sans succès, au moyen de notre régime artificiel. Aussi, sans nier la possibilité d'action sur eux de vitamines inutiles aux pigeons, nous croyons



Graphique II. Le trait plein ——— représente le régime complet.
„ pointillé - - - - - - „ „ sans vitamine B.
„ en croix + + + + „ „ „ „ A.

Les pigeons Nos. 50, 51, 52, 53 étaient âgés d'un mois, quand ils ont été mis au régime artificiel. La première partie de leur courbe représente leur croissance à partir de ce moment. Après trois mois, ils ont été mis pendant 15 jours au régime sans vitamine B, après sept mois définitivement au régime sans vitamine A.



N° 1. Pigeon (n° 2) après 18 mois de régime artificiel (voir tableau II).

N° 2. Pigeon (n° 70) âgé de 5 mois au régime artificiel depuis sa naissance, les parents au moment de la ponte étaient, eux aussi, au régime artificiel depuis 4 mois.

N^{os} 3 et 4. Pigeons (n^{os} 50 et 52) mis au régime artificiel complet à l'âge d'un mois, âgés actuellement de 11 mois, et au régime sans vitamine A depuis 5 mois.

nécessaire, avant d'admettre pareille conclusion, de s'assurer que les phénomènes nutritifs ne sont pas, en ce qui les concerne, conditionnés par des influences étrangères qui nous échappent encore.

Conclusions.

1. Une alimentation artificielle composée de caséïne, d'hydrates de carbone, de graisses, de sels et de cellulose, complétée par de l'extrait de levure (0·8 à 0·9 g. minimum de levure sèche) est suffisante, en l'absence de facteur C pour maintenir l'équilibre physiologique du pigeon, assurer la reproduction et permettre la croissance des petits.

2. Le facteur A ne semble pas nécessaire au pigeon adulte.

BIBLIOGRAPHIE.

Benedict and Sugiura (1923). *J. Biol. Chem.* **55.**
Committee of the Royal Society (1919). *J. Physiol.* **52,** 328.
Drummond (1922). *J. Soc. Chem. Ind.* **41,** 125.
Emmett and Peacock (1922). *J. Biol. Chem.* **50**; *Proceed.* 41.
Funk (1915). *J. Biol. Chem.* December.
—— (1916). *J. Biol. Chem.* **27,** 173.
Hart, Halpin and Steenbock (1922). *J. Biol. Chem.* **52,** 379.
Hoet (1922). *Arch. Internat. Physiol.* **19,** 115.
Kennedy and Palmer (1922). *J. Biol. Chem.* **54,** 217.
Koskowsky (1922). *Arch. Intern. Pharm. Ther.* **26,** 367.
Lipschitz (1923). *Z. physiol. Chem.* **124,** 194.
Medical Research Committee (1919). *Report on accessory food factors,* No. **38,** 50.
Nelson, Lamb and Heller (1922). *Amer. J. Physiol.* **59,** 335.
Osborne (1916). *J. Biol. Chem.* **26,** 5.
—— and Mendel (1921). *J. Biol. Chem.* **45,** 277.
Plimmer and Rosedale (1922). *Biochem. J.* **16,** 11.
Seidell (1922). *Public Health Reports* (U.S.), **37,** 25–1519.
Simonnet (1921). *Bull. Soc. Chim. Biol.* **3,** 10.
Terroine et Barthélemy (1922, 1). *Arch. Internat. Physiol.* **20.**
—— —— (1922, 2). *Compt. Rend. Acad. Sci.* **175,** 721.

XXIX. ON THE THEORY OF GELS. IV.

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From the Science Museum, London.

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The investigation of the physical properties of the natural emulsoids has produced an enormous literature. For the correlation of this, and for the study of many problems of biology and industry a theory of the sol-gel transformation is needed which fits the facts. The present condition of uncertainty is illustrated by the multitude of mutually incompatible *ad hoc* theories of gel structure which are current. The many hypotheses may be classified in three groups: (1) one-phase or molecular systems, (2) two-phase liquid-liquid systems and (3) two-phase liquid-solid systems. To theories in the first class it must be objected that they are unable to explain the loss of mobility that occurs on setting. Those in the second group have the additional difficulty that no liquid-liquid systems can be imagined which would have the elastic properties of gels [Hatschek, 1917]. Nor is there any direct evidence for hypotheses in either of these two categories. Thus we are obliged to assume that gels have a solid phase. This idea is the most natural and was the earliest to be held [Frankenhein, 1835]. The suggestion of K. von Nägeli [1858] is well known, that gels are composed of molecular complexes or micellae with crystalline properties, in the interstices within and between which the water is held by molecular attraction. It has been inferred that von Nägeli intended a geometrical framework, and attempts have been made to devise a network that would account for the elastic and thermal properties of gels. If, however, it is realised both that the elastic properties of gels differ greatly and that the directive forces inherent in the ultimate particles of gels are probably different, it seems unlikely that a single framework could be found to account for the different properties of different gels. It is more probable that the structure of gels varies according to the nature of the gel substance. Bütschli's hypothesis [1892, 1896, 1898, 1900] that gels have a honeycomb structure is disproved by the work of Zsigmondy [1911], Anderson [1914] and Bachmann [1917] on the vapour pressure isotherms of gels, which shows that they must contain fine pores with a radius of from 2·5 to 5μ, some 300 times smaller than Bütschli's honeycombs. These experiments are particularly interesting as they give an explanation of van Bemmelen's [1878, 1880] curious hysteresis cycle. From microscopic work on soap curds and gels, Zsigmondy and Bachmann [1912] have favoured a fibrillar structure. On the other hand McBain [1920] considers that identical colloidal particles are present in the sol and gel

(*Note added on May 17th*, 1923.) Since writing this paper Professor Schryver has very kindly supplied a small sample of specially pure isoelectric gelatin prepared by a new method. This readily yields dilute jellies with marked granular structure in which small spherical particles can be separately resolved, and appears, generally, to behave like the gelatins used for these experiments, except that some doubt has arisen whether large spherites can be obtained under the conditions described.

S. C. B.

state, which differ only in mechanical rigidity and elasticity. Moeller [1916, 1917] supports the view that gelatin gels have a fibrillar structure. But it must be objected to his experiments that the use of alcohol and tanning reagents may alter the structure of the gels or modify the gelation process. Barratt [1920], also, has given evidence that fibrinogen gels are composed of fibrils. For the soaps and fibrinogen there is direct microscopic evidence that fibrils can be formed by the cooling solutions, which suggests that the ultra- or amicroscopic structure of the gels may be fibrillar. For gelatin, agar and silicic acid, however, a globulitic structure is indicated. In gelating 2 % gelatin, submicrons develop gradually from 4, showing Brownian movement, in a square division of the field with a side of 9μ, to 80 to 100 at rest in the same area [Menz, 1909]. Moreover the ultramicroscopic appearance of gelatin gels deprived of water is globulitic [Bachmann, 1911].

Thus there is much evidence that gels contain a solid phase. But none of the theories mentioned is sufficiently definite to explain the reversible sol-gel transformation, or to permit the deduction of the physical properties of gels. Nor do they suggest why such substances as gelatin and agar should occur almost invariably in the colloid form. If it be granted that gels have a solid phase, the origin of the solid must be investigated. The simplest assumption, that the solid was originally in solution and has crystallised out, must be considered first. Here we enter the realm of the theory of solutions, which, unfortunately, is in an altogether unsatisfactory state. Little is known of the forces which hold substances in solution or cause their deposition. Nor is current theory able to suggest the cause of the different solubilities and solvent powers of different substances. Some attempt to study the forces acting on solvent and solute has been made [Bradford, 1919, 1922]. But a subject so vast needs many workers. When these relations are understood, it may be possible to deduce a sound theory of the stability of colloid solutions.

Fortunately, however, von Weimarn's long series of experiments [1914] have thrown some light on the conditions governing the separation of solid from solution. His formula

$$N = U\frac{P}{L}$$

expresses a relation between N, the "form coefficient" of the precipitate, and U, P and L, respectively functions of the viscosity of the reaction medium together with the size and structure of the particles in solution, the excess concentration of the substance to be precipitated, and its solubility. From a great many experiments von Weimarn was able to show that, as N increases, the precipitate passes through stages in which it appears as (1) large complete crystals only after some years, (2) ordinary crystals in a short time (3) growth figures or needles, (4) amorphous precipitates frequently showing microscopic spherical grains, and (5) as a gel which cannot be resolved by the microscope. The colloid condition results when N is extremely great. If, at the same time,

the excess concentration, P, is small, a sol is produced; if P is great, a gel is formed. The formula is not exact. Experiments in progress may help to a more definite statement. But, in practice, the incomplete equation is very useful in showing how to vary the grain size of a given material. The formula suggests directly that gelation is merely an extreme case of crystallisation and that gelatin and agar are substances whose properties lead naturally to a high value of N [Bradford, 1917; 1918; 1920, 1, 2; 1921]. This hypothesis is abundantly confirmed by experiment.

Gelatin sols behave exactly as supersaturated solutions. Certain curious temperature relations of the sols can be simply explained only on the assumption that gelatin has a definite solubility at a given temperature. Moreover, by reducing the value of N in von Weimarn's formula, the particles of gelatin gels can be increased in size as desired and are found to be definitely crystalline.

The molecular theory of solution [Bradford, 1919, 1922] shows that every substance must have a definite solubility, however small, in a given solvent, with which it does not combine chemically. Consider the case of a hot solution of a substance, of high molecular weight and very low diffusivity, in a liquid, in which its solubility, L, is considerable at high temperatures but small at low.

On cooling, the Noyes-Nernst formula,

$$V = \frac{D}{\delta} S (C - L),$$

shows that the velocity of crystallisation will be small, owing to the very small value of the diffusion coefficient, D. The solution will become gradually more and more supersaturated and the particles of solute increasingly aggregated. The value of L in von Weimarn's formula will diminish rapidly, while P increases correspondingly. When, eventually, spontaneous crystallisation occurs, U will be large on account of the high molecular weight and aggregation, P also will be large and L small, giving a maximum value of N. Therefore, the solution will set slowly to a jelly.

A substance with greater solubility, such as oxy-haemoglobin, or egg albumin, will be obtained as micro- or macroscopic crystals even though its molecular weight be very high. But it can be shown that gelatin corresponds exactly to the case considered above. Its molecular weight is not known. However, Dakin's recent analyses [1920] show that gelatin contains 1·4 % of phenylalanine of molecular weight 165. Since one molecule of gelatin cannot contain less than one molecule of phenylalanine the lowest possible molecular weight of gelatin is 11,800. Loeb's [1922] osmotic pressure experiments indicate twice this figure. It is safe to conclude, therefore, that gelatin has a very high molecular weight. A value of 10,000 would correspond to molecules themselves of colloid size with a diameter of 1·75 μ.

The solubility of gelatin was determined in the course of experiments to show that it behaves exactly in accordance with von Weimarn's formula. Dilute filtered solutions of ashless gelatin, purified by electrolysis, from the

Osmosis Co., of different strengths, which had been quickly boiled and sealed in tubes, were allowed to stand at room temperature. Instead of setting to a jelly the solutions became opalescent and the excess of gelatin was deposited slowly as a gelatinous precipitate. When the volumes of the precipitate were plotted against the concentrations, the curve was found to be a straight line cutting the concentration axis at the point corresponding to 0·12 % of dry gelatin per 100 cc. of solution. Above this concentration the volume of the precipitate is proportional to the excess concentration. This figure is undoubtedly the true solubility of gelatin at room temperature, which remained fairly uniform at 18° during the experiment.

Confirmation was supplied by making up solutions of 0·12, 0·13 and 0·135 % and allowing to stand as before. The first remained perfectly clear, the second developed in a few days a beautiful clear bluish opalescence which persisted for months. This was evidently a metastable solution of gelatin, the permanence of which was due to the low diffusion constant of gelatin. With the 0·135 % solution the metastable limit was overstepped and a trace of gelatinous precipitate was deposited. Further confirmation is afforded by the fact that water stirred with gelatin for two hours takes up 0·114 g. of solid. The experiments are being continued with ashless gelatin in a thermostat at different temperatures, and the solubility is being calculated, also, from the amount of solid left in solution in the mother liquor. The results leave no doubt that gelatin has a true solubility in water which increases rapidly with rise of temperature. The high molecular weight is sufficient to account for a very low value of the diffusion constant. Thus gelatin fulfils all the conditions which have been assumed in the hypothetical case considered above. It follows that von Weimarn's formula is sufficient to explain the occurrence of gelatin in the colloid state and that the gelation of gelatin sols is an extreme case of crystallisation.

The theory requires that there shall be a gradual increase in the size of the deposited solid gelatin particles with decrease in supersaturation, until, with very dilute solutions, the precipitated particles become visible microscopically. This is exactly what happens. Six per cent. gelatin gels, from which the liquid has been expressed, cannot be differentiated in the ultra-microscope [Bachmann, 1911]. With less concentration a globulitic structure becomes apparent, and the size of the granules increases as the concentration is reduced. When the concentration is reduced to 0·4 % microscopic examination of the white gelatinous precipitate gives the appearance of an enormous number of particles like grains of sand, just too small to be differentiated, among which there are a number of larger spherical particles big enough to be seen. These are mostly aggregated into clusters. With further decrease in concentration the sand-like appearance becomes more marked and the larger spherical particles fewer and bigger, until, at 0·2 % they reach nearly 1 μ in size. When a gelatin solution is cooled, the bulk of the precipitate increases with the concentration and the particles decrease in size until, at about 0·7 % at 18°

the precipitate fills the solution and forms a continuous jelly. This jelly is white and opaque owing to the large size of the particles. As the concentration is increased the particles get smaller and eventually the jelly becomes clear. A gelatin jelly is, therefore, a gelatinous precipitate of gelatin of more than 0·7 % concentration. The relative sizes of the grains can be determined also in another way. Solutions of ashless gelatin, containing from 0·25 to 1·0 % of dry gelatin, were boiled and sealed in test-tubes and allowed to stand at room temperature. Within 24 hours the solutions up to 0·51 % had become distinctly opalescent, 0·77 % solutions had set to very cloudy just coherent jellies and the 1·0 % tubes contained a slightly stronger jelly that was still rather more cloudy. In about three weeks, the jellies had separated a film of clear liquid above. In the remaining tubes, containing the weaker solutions, the opalescence developed from day to day, and, after about ten days, the precipitate appeared to be subsiding in the tubes. The rate of fall was measured from time to time, and the curious fact was observed that there are three distinct stages in the fall of the surface of the opalescence: (1) a long gradual fall at a uniform rate, for from about 20 to 30 days according to the concentration, during which time the particles are discrete, because the surface of the opalescence is easily disturbed, (2) a sudden drop, for one or two days, after which the precipitate appears to have coalesced to a continuous jelly, and (3) a gradual shrinkage, at a somewhat slower rate than (1), which shows all the signs of syneresis. The application of Stokes's law to the long continuous free fall, (1), enables the relative sizes of the particles to be calculated, and confirms the fact that particles increase in size with diminishing supersaturation. These experiments are being continued in a thermostat at different temperatures, from which it is hoped to determine a more exact relation between the size of the precipitated particles and the solubility and excess concentration. However, each series takes several months, and the solubility increases rapidly with rising temperature, so that a preliminary series is necessary at each stage. Consequently, with very little opportunity for experimental work, it will be some time before the complete results can be published. Similar experiments with dialysed agar give exactly comparable results.

There is no doubt that the solubility of gelatin, like that of any other substance, varies with temperature and with the presence of other bodies. As an amphoteric compound, it is particularly sensitive to changes in the p_{H}. This is confirmed by Loeb's experiments on the rate of solution of gelatin in the presence of salts, acids and alkalis. If, therefore, a solution of gelatin contain more of this substance than corresponds to its solubility under the conditions of the experiment, the excess will be deposited slowly. The gelatin molecules will aggregate gradually in the process of precipitation, and the properties of the liquid will be affected by the presence of these aggregates. Experiments on the viscosity of gelatin solutions compelled Loeb [1922] to assume that the forces holding gelatin and crystalloids in solution are the

same. The viscosity of gelatin solutions is essentially different from that of simple amino-acids or of egg albumin. It is of an altogether higher order, and is much affected by the presence of electrolytes. If it be assumed, as experiments indicate, that the viscosity of gelatin and albumin solutions is a function of the volume of the protein in solution, gelatin solutions must possess a mechanism, which albumin solutions do not, of increasing the volume of the solute. This mechanism is likely to be connected with their tendency to set to a jelly. From which it may be concluded that, in gelatin solutions, solid particles of gelatin, or micellae, are formed gradually, which occlude relatively large quantities of water, whereby the relative volume of the particles is increased.

In egg albumin solutions these particles are lacking because the solutions do not set, *i.e.* the solubility is greater. But if the p_H is below 1·0 and the temperature higher, solutions of albumin do tend to gelate, and then their viscosity is of the same order as that of gelatin solutions.

The influence of electrolytes on the viscosity of gelatin solutions, and not of albumin solutions, is explained by the presence of a Donnan equilibrium between the submicroscopic gelatin particles and the surrounding solution, which regulates the amount of water occluded. This was confirmed by experiments on the viscosity of suspensions of finely powdered gelatin in water, whose viscosity varied directly with the observed volume of the particles, and was influenced by electrolytes exactly as are solutions of gelatin. The effect of electrolytes on the viscosity of gelatin solutions would be explained if it could be shown that they contain submicroscopic particles of solid gelatin capable of swelling. Loeb gives much indirect evidence that solid particles are formed in gelatin solutions, under given conditions, when the temperature falls below a certain point. For instance, the viscosity of 2 % gelatin at p_H 2·7 increases rapidly on standing at temperatures below a point which lies between 25° and 35° and which the experimental values indicate to be at about 27° to 28°. Above this point the viscosity decreases slowly at rates which increase slightly with rising temperature. That is to say a 2 % solution of gelatin at p_H 2·7 is saturated at about 27° or 28°. Below this temperature the excess of gelatin gradually forms solid aggregates, and at higher temperatures the aggregates are gradually dissolved. The actual proof is supplied by direct experiments on the crystallisation of gelatin which show that supersaturated gelatin solutions do deposit solid gelatin.

It follows that the terms "melting point" and "setting point" have no exact meaning as usually applied to gelatin jellies and solutions. A 1·15 % jelly of ashless gelatin will just dissolve completely if it be maintained at 26·45°. If it be kept at a lower temperature, an amount of gelatin, corresponding to the solubility of gelatin at that temperature will go into solution. But, if the jelly be warmed gradually until melted, it will not melt until the temperature has been raised considerably above 26·45° on account of the slow velocity of solution. Conversely, if a warm 1·15 % solution of gelatin

be cooled, the liquid will not set until the temperature has fallen much below 26·45°, or actually to the room temperature. If the temperature be maintained at a point below 26·45°, the gelatin in excess of the solubility at that temperature will be precipitated. Whether the precipitate will form a jelly, or not, will depend on its amount. Obviously, the observed melting and setting points depend on the rate of heating. Similarly the grain size of the jelly is affected by the temperature at which the bulk of the excess gelatin is precipitated. When a more exact expression for the grain size has been found, it should be possible to deduce the relations between the excess concentration, and the elasticity, vapour pressure and other properties of the resulting gels.

It remains to investigate the nature of the deposited solid. It has been mentioned that, when the particles of gelatin became large enough to see, they are spherical in shape. Von Nägeli observed the beautiful brushes shown by spherites between crossed nicols and described their radiating-needle structure, but he was unable to say whether his micellae were identical with spherites. However, it is now certain that gelatin particles have this conformation. The study of substances which crystallise in this shape, and experimental investigation, show that bodies of high molecular weight, and those that exist in solution in the aggregated state, tend to crystallise in the form of spherites. Indeed, it is obvious that a slow moving, comparatively large particle, is able to orientate itself to fit a growing spherite, composed of radiating needles, more easily than a crystal of another shape. It is reasonable to suppose, therefore, that the granules composing jellies of gelatin and agar are spherites.

Spherites are known in every gradation, from the obviously crystalline form, built up of coarse radiating needles separately visible, through stages showing only a more or less radiating formation and often a banded structure, but giving the well-known shadow-cross in polarised light, to apparently isotropic bodies giving little definite evidence of crystalline structure. Microscopic examination of gelatin spherites proves that they are crystalline and belong to the second class. This completes the evidence that gelation is a crystallisation process.

Granules deposited from dilute solutions of pure ashless gelatin under aseptic conditions may reach 1 μ in size. These show perfect shadow crosses when viewed between crossed nicols. It is convenient, however, to grow them larger. This is done exactly in the same way as larger particles of other substances would be grown, *i.e.* by spontaneous evaporation of saturated solutions. In these experiments 0·3 % solutions were made in water containing 0·1 % of mercuric chloride or camphor as antiseptic. The liquid was filtered into chemically clean glass crystallising dishes, which were covered and allowed to stand at room temperature for periods of from one to six months. The liquid becomes opalescent within 24 hours as the precipitation of the gelatin begins. Soon a white, or slightly buff-coloured, powder is seen on the base of the dish. The tint may be due to a trace of pigment, or be the actual colour of the gelatin

particles. Smaller particles always appear white, but it is possible that this may be caused by the greater subdivision. After some time the supernatant liquid is observed to have set to a jelly. This is because evaporation has taken place faster than the gelatin could diffuse through the liquid to the growing spherites. When this happens, further diffusion is practically stopped and the spherites can increase in size only by actual increase in concentration of the liquid in their immediate vicinity, or at the expense of smaller adjacent particles. Consequently the spherites attain nearly their maximum size in a month or two. The precipitate was scraped from the base of the dishes and mounted either in mother liquor, the refractive index of which differs but little from that of the gelatin particles [Walpole, 1913], or glycerol of much higher refractive index, but the spherites appeared to dissolve slowly in the latter medium. The edges of the cover glasses were cemented with Japan gold size. After two months the precipitate consisted entirely of spherites up to 3μ in diameter with a few rather larger grains. They were viewed with a Leitz fluorite $\frac{1}{12}$ inch objective and an eyepiece magnifying twelve times. Between crossed nicols many showed perfect shadow crosses, but, of course, only one or two could be brought into focus at a time. After three months the spherites were up to 3μ or slightly over. There were also a few spherites from each of which a bundle of fibrils had grown like the tail of a comet. Similar appendages are shown occasionally by spherites of silver dichromate. After four months' evaporation, the particles had grown to about 4μ. By six months the larger particles had reached $4{\cdot}4\mu$ in size. Almost all showed shadow crosses more or less complete. But the crosses were the more perfect the smaller the size of the particles. This gives no reason for supposing that the still smaller particles of jellies are not crystalline. The appearance of the precipitate after two months' growth is shown in the photographs (Figs. 1 and 2, Plate III), for help in taking which the writer is indebted to the kindness of Dr L. C. Martin. The shadow crosses show best when the equators of the particles are exactly in focus, and are altogether different in appearance from those shown by light polarised by reflection at the surfaces of air bubbles. Grains of mastic grown by Perrin's method showed no shadow crosses but only a slight luminescence. By evaporation at 23·5° spherites have since been obtained up to 6μ. This proves that the gelatin spherites are crystalline.

Since agar also separates from solution in the form of granules, it appears probable that the structure of jellies of this substance and of gelatin is that of a pile of shot. Such a fine grained structure is compatible with all the known properties of jellies except the heat of swelling, 5·7 cal. per g. [Wiedemann and Lüdeking, 1885] and the so-called "thermal anomaly" [Bjerkén, 1891]. Re-determination of the former gave a value 33·25 cal., which corresponds to that for starch. Investigation of the thermal anomaly showed that it was unfounded. The pile-of-shot structure offers a simple explanation of the lens-shaped form of gas bubbles in gels investigated

by Hatschek [1914]. Moreover, the crystallisation theory explains another curious phenomenon observed by Hatschek [1920]. It is well known that mechanical strain causes gelatin gels to exhibit optical anisotropy in polarised light. When a rectangular prism of jelly is bent between three stops, the usual coloured appearance is observed in polarised light. After five days in a moist chamber, the mechanical stress disappears, so that the specimen can be removed without straightening itself appreciably. Extraordinarily enough, however, the appearance in polarised light remains unchanged. The explanation may be as follows. Gelatin and agar are remarkable for their stability, although most substances that can be obtained in the gel form, under suitable conditions, develop rapidly microscopic crystals. The permanence of the natural gels is due to the low solubility, the very low rate of diffusion of the gel substances, and to the fact that dissolved material is almost unable to diffuse through the jelly. But, if the crystallisation theory is true, very slow re-crystallisation should be going on. Evidences of slow re-crystallisation in silicic acid gels have been observed previously. They have now been obtained in the case of gelatin.

Optically clear aseptic 5 % gelatin gels were sealed in tubes and kept at room temperature. After three months a distinct opalescence had developed, which grew from month to month until, after ten months, it had become very marked. Microscopic examination after four months showed an almost clear field in which some very tiny round particles could just be seen. With lapse of time the visible particles increased in number and size. In ten months some had reached nearly 1μ in size. It appears, therefore, that there is an extremely slow process of re-crystallisation taking place constantly in gelatin jellies, by which the larger particles are growing continually at the expense of the smaller ones, just as in the case of any ordinary substance in contact with a solvent. Moreover, the marked increase in the modulus of elasticity during the first 24 hours indicates that the separation of gelatin from solution, in the slow process of setting, is not complete even within that time. Since gel substance is being deposited on the larger particles in a gel, whether strained or not, in a strained gel the larger particles will become cemented in their constrained positions. So that, on removal of the stress, the particles will be unable to move, but the internal strain will continue and maintain the optical appearance in polarised light.

In default of evidence to the contrary, therefore, we must conclude that the forces which hold gelatin and agar in solution are the same as those that operate between other solutes and solvents, and that the setting of jellies of the natural emulsoids is merely a process of crystallisation.

The writer desires to express his gratitude to the Government Grant Committee of the Royal Society and to the Chemical Society for grants which have enabled these experiments to be continued.

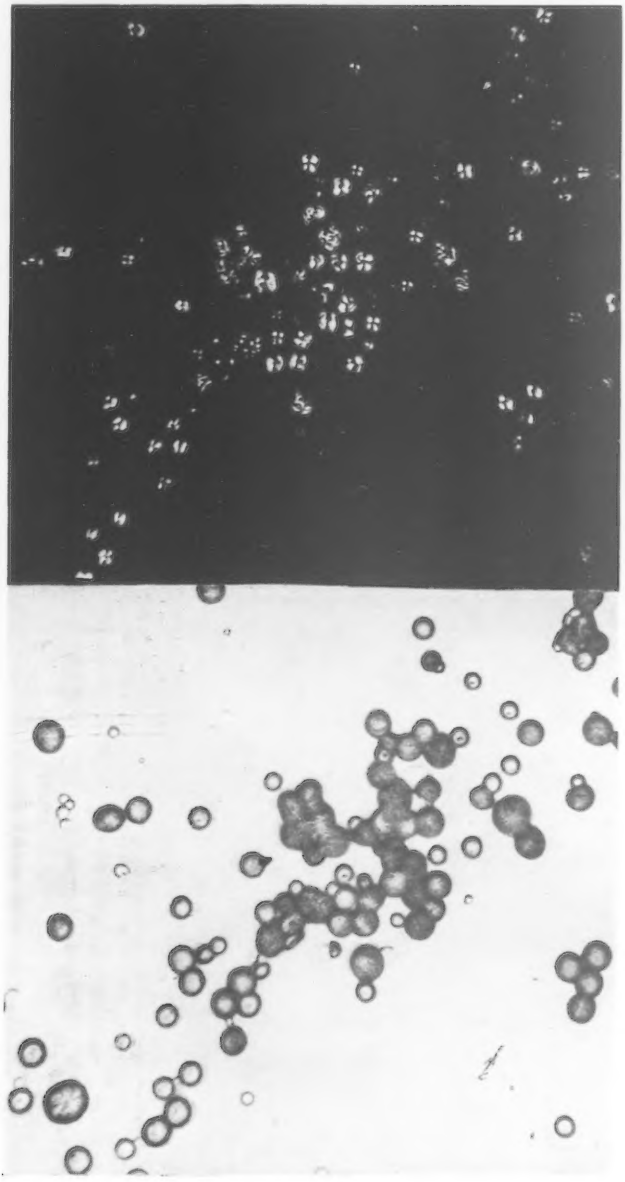

Fig. 2. Gelatin spherites between crossed nicols. ×1400

Fig. 1. Gelatin spherites. ×1400.

REFERENCES.

Anderson (1914). *Ann. Chim. Phys.* [vi], **29**, 505.
Bachmann (1911). *Z. anorg. Chem.* **73**, 150.
—— (1917). *Z. anorg. Chem.* **100**, 1.
Barratt (1920). *The physics and chemistry of colloids.* A discussion by the Faraday and Physical Society, 1921.
Bemmelen (1878). *Ber. deutsch. chem. Ges.* **11**, 2232.
—— (1880). *Ber. deutsch. chem. Ges.* **13**, 1466.
Bjerkén (1891). *Ann. Phys. Chem.* **43**, 819.
Bradford (1917). *Science Prog.* **12**, 63.
—— (1918). *Biochem. J.* **12**, 351.
—— (1919). *Phil. Mag.* **38**, 696.
—— (1920, 1). *Biochem. J.* **14**, 91.
—— (1920, 2). *The physics and chemistry of colloids.* A discussion, 1921.
—— (1921). *Biochem. J.* **14**, 553.
—— (1922). *Phil. Mag.* **44**, 897.
Bütschli (1892). Untersuchungen über mikroskopische Schaume, Leipzig.
—— (1896). Über den Bau quellbarer Körper, Göttingen.
—— (1898). Untersuchungen über Strukturen, Leipzig.
—— (1900). Untersuchungen über den Mikrostruktur...Kieselsäure-gallerten, Heidelberg.
Dakin (1920). *J. Biol. Chem.* **44**, 499.
Frankenhein (1835). Die Lehre von der Kohäsion, Breslau.
Hatschek (1914). *Koll. Z.* **15**, 226.
—— (1917). *Trans. Farad. Soc.* **12**, 17.
—— (1920). *The physics and chemistry of colloids.* A discussion, 1921.
Loeb (1922). Proteins and the theory of colloidal behaviour, New York.
McBain (1920). *The physics and chemistry of colloids.* A discussion, 1921.
Menz (1909). *Z. physikal. Chem.* **66**, 137.
Moeller (1916). *Koll. Z.* **19**, 205.
—— (1917). *Koll. Z.* **20**, 257.
Nägeli (1858). Pflanzenphysiologische Untersuchungen, Heft 7, Zurich.
Walpole (1913). *Koll. Z.* **13**, 241.
Weimarn (1914). Zur Lehre von den Zuständen der Materie, Dresden.
Wiedemann and Lüdeking (1885). *Ann. Phys. Chem.* **25**, 147.
Zsigmondy (1911). *Z. anorg. Chem.* **71**, 356.
—— and Bachmann (1912). *Koll. Z.* **11**, 145.

XXX. THE BALANCE OF ANIONS AND KATIONS IN THE PLASMA IN NEPHRITIS.

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This work was begun with the intention of finding what anions, accumulated in the plasma, were responsible for the occurrence of acidosis, as shown by diminution of plasma [HCO_3^-], in patients with nephritis. Such an accumulation of anions might be due either to a general impairment of excretion resulting in the retention to a greater or less degree of all the anions which should be excreted in the urine, or to a specific inability to excrete some particular anion resulting in the retention of this anion alone.

In order to decide to what degree any particular anion is responsible for a diminution of plasma [HCO_3^-] it is necessary to know the concentrations of all the anions and kations in the plasma; for an increase of one anion will not cause an acidosis if accompanied by a corresponding increase of kation or diminution of some other anion. This explains the lack of correspondence found for example by Denis and Minot [1920] between accumulation of phosphate in the plasma and diminution of plasma bicarbonate.

It is therefore necessary to calculate the balance between the anions and kations in the plasma of patients with acidosis, and for purposes of comparison in that of other patients.

In making this balance other points with regard to individual constituents of plasma and the balance between them have arisen and I will consider these besides the original question of the cause of acidosis.

Table I. *Balance of anions and kations in plasma of control cases.*

Anions.

Average of control cases	[HCO_3^-]	—cc. of CO_2 per 100 cc.	61·0	0·0277 g. equivalents per litre			
	[Cl^-]	—g. NaCl	,,	0·600	0·1026	,,	,,
	[HPO_4^{--}]	—g. P_2O_5	,,	0·0066	0·00165	,,	,,
Denis [1921]	[SO_4^{--}]	—g. S	,,	0·0008	0·0005	,,	,,
	Protein	—and organic acids			0·0105	,,	,,
				Total	0·143		

Kations.

Average of control cases	[Na^+]	—g. Na	per 100 cc.	0·030	0·1305 g. equivalents per litre		
Myers and Short [1921]	[K^+]	—g. K	,,	0·020	0·005	,,	,,
	[Ca^{++}]	—g. Ca	,,	0·010	0·005	,,	,,
	[Mg^{++}]	—g. Mg	,,	0·003	0·0025	,,	,,
				Total	0·143		

Table II. *Balance of anions and kations in cerebrospinal fluids from patients with no evidence of nephritis. These fluids contained only minute traces of protein.*

Anions.

Average of these cases	$[HCO_3^-]$	—cc. of CO_2 per 100 cc.	62·0	0·028 g. equivalents per litre		
	$[Cl^-]$	—g. NaCl	,,	0·735	0·126	,, ,,
	$[HPO_4^{--}]$	—g. P_2O_5	,,	0·004	0·001	,, ,,
				Total 0·155		

Kations.

Average of these cases	$[Na^+]$	—g. Na	per 100 cc.	0·337	0·147 g. equivalents per litre	
Myers [1917]	$[K^+]$	—g. K	,,	0·022	0·005	,, ,,
Halverson and	$[Ca^{++}]$	—g. Ca	,,	0·005	0·0025	,, ,,
Bergeim [1917]	$[Mg^{++}]$	—g. Mg	,,	0·003	0·0025	,, ,,
				Total 0·157		

The figure given for Mg in cerebrospinal fluid is that for plasma as I can find no estimate of magnesium in cerebrospinal fluid.

Balance of anions and kations in normal plasma.

The balance of the average values of anions and kations in my control cases is shown in Table I. There is an excess of kations unaccounted for by Cl^-, HCO_3^- and HPO_4^{--} equal to about 0·010 g. equivalents per litre. This is combined with protein and anions of organic acids. Probably organic acids only account for a small fraction of these kations. In cerebrospinal fluid which contains practically no protein, the excess of kations does not occur, as shown in Table II. Also Greenwald and Lewman [1922] have recently published a method by which they estimate the amount of kations in plasma combined with HCO_3^- and with protein together. In the normal cases in which the bicarbonate is given, the kations combined with protein are equivalent to 0·0097 and 0·0136 g. equivalents per litre, and are therefore about equivalent to the amount of base in plasma unaccounted for by HCO_3^-, Cl^- and HPO_4^{--}.

Kramer and Tisdall [1922] have published a table of average values of the anions and kations in plasma showing the excess of bases unaccounted for as 0·025 g. equivalents per litre. I think it probable that the "average" value taken by them for plasma $[Na^+]$ is high in proportion to their "averages" for $[Cl^-]$ and $[HCO_3^-]$.

I have estimated only the $[HCO_3^-]$, $[Cl^-]$, $[HPO_4^{--}]$ and $[Na^+]$. Calcium may vary between 0·006 and 0·011 %, or between 0·003 and 0·0055 g. equivalents per litre, potassium between 0·010 and 0·020 % or between 0·0025 and 0·005 g. equivalents per litre. Variations of magnesium are negligible as the amount is so small. If, therefore, we add to the $[Na^+]$ 0·0125 g. equivalents we shall obtain an estimate of the total kations with a maximum error of 0·0045 g. equivalents per litre, not more than 3·5 % of the total kations, and little more than the errors in sodium estimation.

As normally the principal anions, HCO_3^-, Cl^- and HPO_4^{--}, are about equivalent to the Na^+, and therefore the other kations to the normal protein, it is possible to omit these last items in balancing the anions and kations.

Taking them into account merely involves adding two equal amounts to both sides of the equation.

In such a balance an accumulation of Cl^- or HPO_4^{--} in the blood, causing a diminution of plasma bicarbonate, is shown directly; while, if an accumulation of another acid ion, such as SO_4^{--}, occurs, its presence is shown by an excess of Na^+ unaccounted for by the anions estimated. Such an accumulation is illustrated in Table III; the excess of Na^+ is equivalent to the keto-acids within the limits of error of the method.

As I originally intended to consider acidosis only, the patients in this series have been selected mainly from severe cases of nephritis, who might be expected to have some disturbance of their acid base regulation.

Table III.

Excess of Na^+ unaccounted for by HCO_3^-, Cl^-, and HPO_4^{--} in plasma in ketosis.

Case	p_H	$[HCO_3^-]$	$[Cl^-]$	$[HPO_4^{--}]$	Sum of three anions	$[Na^+]$	Excess Na^+	β-Hydroxy-butyric acid and aceto-acetic acid	
		Gram equivalents per litre							
60	7·0	0·0055	0·112	0·002	0·1195	0·144	0·0245	0·0215	Diabetic coma; Moderate hyperpnoea. Blood sugar 0·44 %. Died next day.
61	7·4	0·0215	0·099	0·002	0·1225	0·1305	0·008	0·0065	Diabetes; prolonged ketosis without coma. Blood sugar 0·26 %.

Total acetone bodies were estimated by the method of Van Slyke; 20 % was assumed to be present as acetone.

Some cases of heart failure have been included to illustrate the changes that may be found in this condition.

Methods.

For plasma bicarbonate when determined without p_H Van Slyke's [1919] titration method was used. When p_H and plasma bicarbonate were determined, methods of Van Slyke [1922] and Cullen [1922].

The blood was obtained through a needle with a short piece of rubber tubing attached; a piece of rubber tubing was fixed tightly round the patient's upper arm. After the needle was inserted the blood was allowed to flow, to allow all congestion from the constriction of the arm to pass off, and the tubing was then connected to the entry tube of a 25 cc. centrifuge tube as shown in Fig. 1. By withdrawing the wide part of this entry tube a short distance from the hole in the stopper, air was allowed to escape as the blood flowed in. The centrifuge tube had previously been filled with alveolar air in order that the CO_2 tension of the oil and any air with which the blood came in contact might approximate as nearly as possible with that of the blood taken. When the oil began to overflow the entry tube was removed, a glass plug inserted,

and the tube inverted three or four times. The effect of the small bubble of air left in the tube would be negligible. The blood was then rapidly centrifuged and the plasma thus obtained was removed with a pipette through the hole in the stopper for p_H, bicarbonate, Cl and P_2O_5 determinations. These therefore were determined at the CO_2 tension of the patient's venous blood.

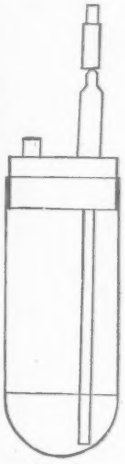

Fig. 1.

The solutions of known p_H used for p_H determinations were made according to the formula in Cullen's paper, from pure salts recrystallised. These solutions contained the theoretical amount of phosphorus determined gravimetrically. The p_H is given to the nearest 0·05 as it was occasionally difficult to get a more exact match.

Chlorides were estimated by the Volhard method on filtrates after precipitation of protein by trichloroacetic acid. Precipitation with trichloroacetic acid is less complete than with picric and tungstic acids, and in some cases some difficulty was experienced in getting the silver chloride to flocculate satisfactorily, probably owing to proteoses left unprecipitated. In two cases, in one of which the flocculation did not take place readily and in the other the chloride value was very low, the result was checked by an incineration method.

In some cases, when sufficient blood was not available for estimation of chlorides in whole blood, the corpuscles separated from the plasma as above were used for chloride determination. As the separation was done rapidly it is probable that the corpuscles were not completely separated from plasma and that the Cl values found are too high; this would occur especially in the more normal cases in which the corpuscles sink slowly. These figures therefore are upper limiting values only.

Inorganic phosphates. The figures given in brackets were estimated by the original method of Bell and Doisy [1920] before the effect of oxalate was realised. The amount of oxalate used was minimal, and the figures are therefore probably a little too low. Later the modification given by Denis and Meysenbug [1922] and the method of Briggs [1922] were used.

Sodium was estimated by the method of Kramer and Tisdall [1921]. This method gave theoretical results with pure solutions of sodium chloride, duplicates agreed and added sodium chloride was satisfactorily recovered from plasma or serum.

Determinations were mainly made on plasma as more can be obtained from a given amount of blood, and the delay and risk of haemolysis during the separation of serum is avoided by using plasma.

Determinations on plasma and serum from the same blood agreed.

This method is liable to an error of $\pm 3\,\%$ which is of importance in considering the balance of acids and bases.

Calcium was determined in a few cases by the method of Clark [1921].

Plasma proteins were determined in some of the cases by Dr Villett by the method of Wu [1922, 2]. He has kindly permitted me to use his figures.

Blood urea was estimated by the urease method.

In Table V [HCO_3^-], [Cl^-], [HPO_4^{--}], and [Na^+] are given in g. equivalents per litre; "Sum" represents the sum of the g. equivalents per litre of the anions.

Chlorides in plasma.

The concentration of Cl^- in plasma is affected by changes of the tension of CO_2 which cause a migration of Cl^- between the red corpuscles and plasma, but the changes produced thus are much less than the variations found in these cases. Thus Doisy, Eaton and Chouke [1922] found the plasma [Cl^-] fall from 0·65 to 0·61 % NaCl (0·111 to 0·104 g. equivalents per litre), in human blood with an increase of CO_2 tension from 23·3 to 72·5 mm. These values for CO_2 tension are extreme; such deviations from normal CO_2 tensions do not occur in normal persons, and were only found in the extreme cases in this series. In normal persons, also, variations of plasma [Cl^-] occur much greater than those caused by variations of CO_2 tension. Thus in the series given by Wu [1922, 1] the values ranged from 0·56 to 0·67 % NaCl—0·096 to 0·1145 g. equivalents per litre. His figures for the Cl in the corpuscles also show that the variations in plasma were not due to migration of Cl^- in and out of the corpuscles, as high concentrations in plasma were usually associated with high values in corpuscles and *vice versa*.

The variations found by Wu are greater than those usually found, *e.g.* by Norgaard and Gram [1921], although Wu and Norgaard and Gram are in fair agreement as to the average value, about 0·62 % or 0·104 g. equivalents per litre.

The plasma Cl in this series was on the whole low. Out of 98 determinations 27 were under 0·096 g. equivalents per litre.

In another series of 100 patients taken without selection of the more severe cases the plasma [Cl^-] was under 0·096 g. equivalents per litre in 7.

All patients with plasma [Cl^-] under 0·092 g. equivalents per litre were severely ill, and those with values under 0·0855 still more so. In cases such as (31), (32), (38) and (39) which steadily became worse the plasma [Cl^-] steadily fell; this did not always occur however, as for example in case (33). Cases (6) and (37) had very low figures when at their worst with an increase on improvement.

There is no relation between the type of nephritis and the plasma [Cl^-]. Oedema occurred both with normal and low values.

There is no evidence that the variations were due to variations of CO_2 tension or reaction of the plasma or to migration of Cl^- between plasma and corpuscles[1]. Thus case (47) with an alkaline plasma had a plasma [Cl^-]

[1] It is not suggested that migration of Cl^- between corpuscles and plasma did not occur, but that it was overshadowed by other changes.

0·093 g. equivalents per litre and the low plasma [Cl^-] of cases (31), (32) and (39) was associated with low values for Cl in the corpuscles.

Phosphates in plasma.

The plasma [HPO_4^{--}] varies in the same sense as the blood urea but not proportionately; which is to be expected from the large and variable excretion of phosphate which takes place in the faeces also. Two cases, (39) and (40), especially have high blood urea with no increase of plasma [HPO_4^{--}]. In these cases Cl appears to have been stored in the tissues and it is possible that a similar storage of HPO_4^{--} occurred; but one cannot exclude the possibility that the excretion of HPO_4^{--} may have been performed completely by the intestine, so that there was actually no retention. It is remarkable that in these two cases the kidneys were entirely out of action.

Sodium in plasma.

Two values for normal sodium concentration in plasma are found in the literature. Those of Kramer [1920] 0·28 to 0·31 %, 0·122 to 0·135 g. equivalents per litre, and those of Kramer and Tisdall [1921], 0·326 to 0·350 %, with average of 0·33%, 0·142 to 0·152, with average 0·144, g. equivalents per litre. My figures for the mild cases of nephritis and cases without evidence of kidney damage agree better with the first.

The values found in this series were on the whole low, but reductions of [Na^+] as great as those of [Cl^-] did not occur. Except in case (37) (10. viii. 22) the reduction of [Na^+] was not more than equivalent to that of [Cl^-] in the same patient.

It is remarkable that, as has long been known for Cl, as is shown by Myers and Short [1921] for K, various workers for Ca and by these results for Na, no accumulation of these constituents occurs in the plasma in nephritis, even with extreme degrees of renal inefficiency, which are accompanied by great increase of plasma urea and HPO_4^{--}. This points to methods of regulation of the amounts of the former substances in the plasma other than excretion through the kidneys; which methods may be retention of water, and deposition in the tissues.

In case (39) the water retention must have been inadequate to keep down the Cl concentration; the NaCl retained during 15 days on ordinary diet with no restriction of Cl intake must have been at least 45 g., which would require 11 litres of water to dilute it to the concentration of the plasma at death; an amount which could not have been retained without gross oedema. The second process therefore, deposition in the tissues, must have played a large part. His Na intake was increased by large doses of sodium bicarbonate, the absorption of which is proved by increase of the plasma bicarbonate to normal, and its subsequent maintenance at normal in spite of increasing amounts of undetermined acids in the plasma; but the plasma [Na^+] did not increase,

instead towards the end it fell. Accompanying this there was a steady fall of [Cl^-]. This suggests that Na also was deposited in the tissues so that, instead of the plasma bicarbonate being increased by the absolute increase of [Na^+] in the plasma, it was increased by removal of Cl^- into the tissues along with the extra base supplied, leaving a relative excess of Na^+ in the plasma.

A similar effect of treatment with bicarbonate is seen in other cases, (13), (26), (30) and (35), although in these we have not such satisfactory evidence that the result is not brought about by water retention; in case (35) especially the output of water during two days of bicarbonate administration fell below the intake by 2 litres, although after the administration was stopped output was equal to intake.

The low values of plasma [Cl^-], and to a less degree of plasma [Na^+], point to some disturbance of the mechanisms that regulate these constituents. It is probable that in most of the cases water retention played a large part; such water retention would explain the low values of cell volume and total proteins[1]. It is probable, however, that tissue deposition, as in (39), occurs also in other cases; in case (31) for example, in which output equalled intake in spite of profuse sweating until the last three days, water retention alone does not seem adequate to explain the fall in plasma [Cl^-] which occurred in spite of retention of about 1 g. of NaCl a day.

There is further evidence of such a deposition of chloride in the tissues in the high Cl content of the heart muscle in cases (39) and (33)—0·24 and 0·31 % of sodium chloride as compared with 0·16 and 0·19 in the hearts of two healthy men who were killed suddenly in accidents. Such deposition in the tissues was shown by Wahlgren [1909] to occur in dogs after large doses of sodium chloride; he found however that the percentage increase in muscle was less than in other tissues, so that had some other organ been chosen for analysis in these two cases the increase found might have been greater. In cases (26), (32), (33) there is a striking increase in the [Cl^-] and [Na^+] in the cerebrospinal fluid in comparison with that in the plasma.

In cases (3), (6), (31), (32) and (38) the excretion of Cl in the urine was continued although the plasma Cl had fallen well below the recognised threshold.

It is reasonable to suppose that these changes are all due to some alteration of the mechanism which regulates the distribution of sodium chloride between plasma and other body fluids. It is possible that this is the result of changes in the plasma proteins.

The low figures found for [Cl^-] and [Na^+] are of great importance in the balance of acids and bases. Such changes make it impossible to calculate what effect accumulation of some acids such as phosphoric will have on the plasma bicarbonate unless these constituents also are estimated.

[1] The change in plasma proteins however is more than a mere reduction of total protein; there is a change in the proportions of the protein fraction and actual increase of fibrinogen.

Balance of acids and bases.

In the cases of hyperpiesia the relation between the sum of the anions estimated and the sodium is the same as in the control cases. In the mild cases of nephritis (2), (4), (6), (14) and (15) there is slightly more sodium in relation to anions but not enough to justify any conclusion. Also in several of the more severe cases, (26) (except on 27. iv. 22), (30), (34) and (38) (on 28. vii. 22) the relation differs little from that in the controls.

Table IV.

Average values in g. equivalents per litre

	$[HCO_3^-]$	$[Cl^-]$	$[HPO_4^{--}]$	Sum	$[Na^+]$
Control	0·0274	0·1026	0·0016	0·1316	0·1305
Hyperpiesia	0·032	0·099	0·002	0·133	0·131
Mild cases	0·029	0·103	0·002	0·134	0·1365

But in 18 cases (1, 9, 12, 13, 18, 23, 25, 26, 28, 29, 31, 32, 33, 35, 39, 40, 41 and 52), the Na exceeded the sum of these three anions by more than 0·0085 g. equivalents per litre, the maximum error to which this method of estimating the total kations is liable. Of these, 16 were frankly severe cases with blood urea raised. Of the remaining two, one case, (52), had severe heart failure, and one case, (18), was an old man with an enlarged prostate and considerable impairment of renal efficiency as tested by phenolsulphonephthalein excretion and urea concentrating power.

A steady increase of this excess of kations is seen in cases (31), (32) and (39) as the blood urea rose, while in cases (6) and (1) the excess of kations fell as the patient improved and the blood urea fell.

This excess of kations may be combined (1) with protein or (2) with undetermined acid ions.

The changes in plasma proteins that occur in nephritis are reduction in total protein and albumin, increase of fibrin and a relative and sometimes absolute increase of globulin. These changes are seen in cases (7), (9), (11), (31), (32) and (33). It is difficult to see how such changes can result in an increase of kations combined with protein unless we suppose the fibrin has a much greater power of combining with kations than have albumin and globulin. In case (32) the excess of kations was much greater when the total protein was 6·04 % than when it was 7·89 %; in case (33) with high fibrin there was no excess of Na^+.

In the cerebrospinal fluids of cases (26), (32) and (33) there is no excess of kations, as in the plasma taken before and after, but the difference between the sum of the three anions and the Na^+ is the same as in normal cerebrospinal fluids. This might be due to the absence of protein, but can be just as well explained by supposing that there is a much lower concentration of the unknown anions in cerebrospinal fluid than in plasma, just as is the case with phosphoric acid, and as found by Berglund [1922] with amino-acids, creatine and uric acid.

It is therefore probable that the excess of kations is not combined with protein but with some anions which have not been determined.

Undetermined acid in the form of keto-acids probably accounts for the excess in case (40) and possibly in the form of lactic acid in cases (26) and (52).

In the other cases it is probable that no one anion is responsible, but a mixture of anions which should be excreted in the urine—among these SO_4^{--} which has been found by Denis [1921] in nephritic blood in sufficient amount to produce this effect.

In the ascitic fluid of case (39), post mortem there were 12 mgm. S as sulphate per 100 cc., or 0·0075 g. equivalents per litre. Such a concentration would account for part of the excess of kations of 0·021 g. equivalents per litre found in the plasma.

However this sulphate may have been produced by bacterial changes which had taken place in the fluid.

It is hoped to evolve a method of sulphate estimation applicable to the small quantities of plasma available.

Plasma bicarbonate and reaction.

Owing to the migration of Cl^- between red corpuscles and plasma with variations of the CO_2 tension, it is necessary to have some method of distinguishing low plasma bicarbonate due to accumulation of acid in the blood from low plasma bicarbonate due to the exposure of an otherwise normal blood to abnormally low CO_2 tensions with consequent migration of Cl^- into the plasma. Charts I and II serve this purpose. Abscissae represent venous CO_2 tension, ordinates $[HCO_3^-]$ in g. equivalents per litre. Since the hydrogen ion concentration of plasma is proportional to the ratio of the CO_2 tension to the plasma bicarbonate, the points representing plasmata with the same hydrogen ion concentration and therefore the same p_H must all lie on a straight line passing through the origin. Such lines for p_H 7·1 to 7·6 are shown; they were calculated by the formula given by Austin *et al.* [1922].

The point representing a plasma can be determined by its $[HCO_3^-]$, giving the ordinate, and p_H, giving the p_H line on which it lies, or CO_2 tension, giving the abscissa.

Points for plasmata separated from normal bloods at varying CO_2 tensions may lie within the shaded area. The two curves shown are taken from the paper of Doisy, Eaton and Chouke [1922] and the limits of the normal area are calculated from the limits of the normal dissociation curves given by Peters, Barr and Rule [1921]. If a low $[HCO_3^-]$ is due to the separation of the plasma from otherwise normal blood at low CO_2 tension, the point for the plasma will still fall inside the normal area, while if it is due to accumulation of anions in the blood (acidosis) the points will fall below the normal area. If there is an excess of kations in the blood (alkalosis) the points will fall above the normal area.

The normal p_H lies between 7·3 and 7·4 the average being nearer 7·4. Normal cases should therefore fall between these two lines.

The plasmata of control cases all fall within the normal area, and with the exception of case (56) have normal p_H. Case (56) is an example of the effects of hyperpnoea reducing the venous CO_2 tension and causing a high p_H and comparatively low plasma $[HCO_3^-]$. A similar condition has been produced outside the body in case (59) by not taking precautions to avoid loss

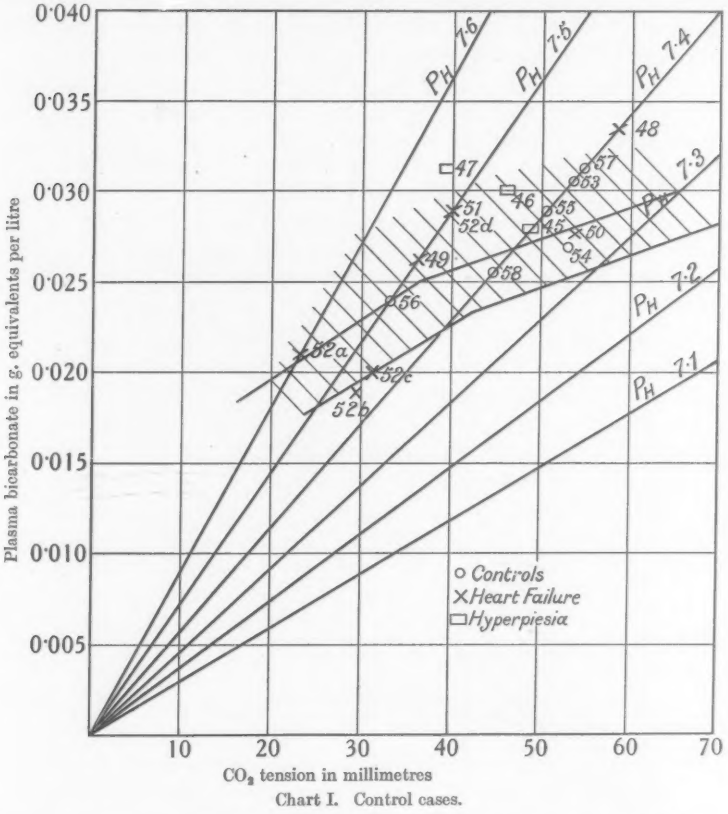

Chart I. Control cases.

of CO_2. In the balance of acids and bases in these two cases we find evidence of the migration of Cl^- into the plasma in the increase of $[Cl^-]$ above the average without corresponding increase of $[Na^+]$. As closely as can be expected with the errors to which the method is liable the fall in $[HCO_3^-]$ is accounted for by this relative increase of $[Cl^-]$.

In the cases of heart failure two (48) and (52) (*b*) and (*c*) fell at the upper and lower limits of the normal area and three (49), (51) and (52) have high p_H.

This high p_H in heart failure which is consequent on a low venous CO_2 tension was also found by Fraser, Ross and Dreyer [1922]. It may be due either to shortage of oxygen leading to the circulation of some products of incomplete combustion other than acid, which stimulate the respiratory centre with resulting increase of lung ventilation, so that CO_2 which passes out of the blood into the alveolar air more readily than O in the reverse direction is washed out of the blood; or to a local stagnation of the circulation in the respiratory centre leading to local accumulation of CO_2 and stimulation of the respiratory centre although the CO_2 tension in the general circulation is being abnormally reduced.

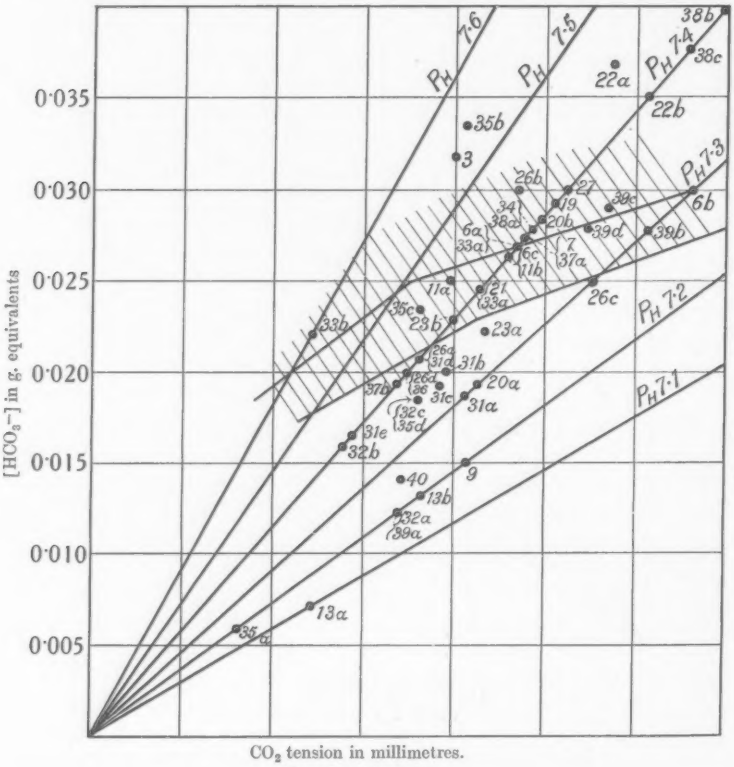

Chart II. Nephritis.

Case (52) affords an example of the variation of p_H and bicarbonate that may occur in heart failure from interaction of the two factors hyperpnoea and acid accumulation. On 4. xi. 22 hyperpnoea had reduced her CO_2 tension with resulting high p_H. On 10 and 11. xi. 22, when much worse, CO_2 elimination was also impaired with the result that the venous CO_2 tension rose in

spite of increased hyperpnoea, while there was an accumulation of undetermined anions resulting in reduction of the [HCO_3^-]; in consequence of these two changes the plasma p_H fell. With great clinical improvement on 14. xi. 22 her [HCO_3^-] rose above the value on 4. xi. 22 but her CO_2 tension remained still somewhat low although the hyperpnoea was hardly noticeable.

In cases (49) and (52) the migration of Cl^- is not detectable, being obscured by other changes.

The plasmata of two cases, (45) and (46), of the three cases of hyperpiesia in which the p_H was determined, fell in the normal area, and of one (47), who had been treated with large doses of sodium bicarbonate, above this area.

Cases (46) and (47) with high p_H had disturbances of the central nervous system which may have affected their respiratory centres. Case (47) was also in considerable distress from violent headache, which may also have caused hyperpnoea. In these two cases the excess of plasma [Cl^-] and low bicarbonate are not seen; in case (47) the absence of high plasma [Cl^-] is explained by the low [Cl^-] in the whole blood, so that even with a lower proportion of this Cl^- than usual in the corpuscles that in the plasma would not be high.

In all the mild cases of nephritis (Chart II) the plasma [HCO_3^-] fell within the normal area. The average [HCO_3^-] for the five cases (2, 4, 5, 14 and 15) was 0·0287 g. equivalents per litre which agrees closely with that for the controls. In one case (15) (pyelitis of pregnancy) the plasma p_H was high. It is not improbable that a slight degree of hyperpnoea and consequent high plasma p_H occurs in pregnancy apart from any pathological condition. In this case as in case (47) the [Cl^-] in the plasma and blood was low.

Of the severe cases also a large number fell in the normal area; among these are cases (26, *b*) (2. v. 22), (33), (34), (39 *d*) (29. vi. 22) with high plasma [HPO_4^{--}] or undetermined anions showing that in these cases kation had been retained in sufficient amount to neutralise these anions. Cases (3), (22 *a* and *b*) (5. vii. 22 and 13. vii. 22) (38 *b* and *c*) (3. vii. 22 and 28. vii. 22) and (35 *b*) fall above the normal area; cases (9), (13), (32), (35 *a*) (16. xi. 22), (39 *a*) (21. vi. 22) and (40) fall very low, and cases (31), (20 *a*) (23. vi. 22), (26 *a* and *d*) (27. iv. 22 and 17. vii. 22), (36), (37 *b*) (10. viii. 22) and (23 *a*) (28. vi. 22) below the normal area.

In the majority the plasma p_H was normal, in (11 *a*) (3. viii. 22), (22 *a*) (5. vii. 22) and (26 *b*) (2. v. 22) slightly raised, and in (3), (35 *b*) (20. xi. 22), and (33 *b*) (26. x. 22) very high. Of these, cases (22 *a*) and (35 *b*) who had received large doses of sodium bicarbonate, had a high plasma [HCO_3^-] not fully compensated by increased CO_2 tension. Case (3) who had also received large doses of sodium bicarbonate had a temperature of 101° accompanied by definite hyperpnoea.

Case (33 *b*) (26. x. 22) had heart failure with extreme dyspnoea and is therefore comparable to the case (52 *a*). On treatment with morphia, which presumably deadened his respiratory centre, the dyspnoea was much reduced and his plasma p_H returned to normal. Other cases of heart failure with

nephritis, cases (21), (23) and (38), do not show a high p_H so that the increased respiratory effort in these cases was ineffective, or had less effect than other changes.

The cases which fell very low in the charts had abnormally low values for plasma p_H—7·1 and 7·2—that is to say their respiratory mechanism failed to reduce their CO_2 tension in proportion to their low plasma $[HCO_3^-]$.

Returning to the cases which fell outside the normal area. Those, (3), (22 *a* and *b*) (5. vii. 22 and 13. vii. 22) (35 *b*) (20. xi. 22) and (38 *b* and *c*) (3. vii. 22 and 28. vii. 22), falling above the normal area had all received doses of sodium bicarbonate which resulted in high plasma $[HCO_3^-]$ levels. In all the plasma $[Cl^-]$ is low and the $[Na^+]$ not increased, that is the high plasma bicarbonate has been produced not by an actual but by a relative increase of kations in the plasma and a reduction of anions in the shape of Cl^-. Case (38 *b* and *c*) had heart failure and oedema of the lungs with very high CO_2 tension compensating his high plasma $[HCO_3^-]$.

The cases falling below the normal limit are the true cases of acidosis. With them may be included cases (12) and (24); in their case the plasma p_H has not been estimated but there is no reason to suppose that they were cases with low plasma $[HCO_3^-]$ consequent upon reduction of the venous CO_2 tension by hyperpnoea. There were, therefore, in this series of 41 cases with definite kidney damage, 14 with acidosis. The large proportion in this series is due to the severity of the cases chosen. In another series of 100 cases which included a larger proportion of mild cases there were 22 with plasma bicarbonate below 55, and these cases may not all have been true cases of acidosis as some of the less low plasma bicarbonates may have been secondary to reduced CO_2 tension only. The severity of the condition is shown by the fact that of these twenty-two, out of five who had plasma bicarbonate between 35 and 44, four died within two weeks, and out of the five who had plasma bicarbonate below 35 all died within a few days. This heavy mortality also occurred among the cases considered in this paper; of the six cases with severe acidosis four died within fourteen days, and one within a month.

By comparison of the anions and kations in the plasmata of these cases with those of the control cases, we find that the diminution of plasma $[HCO_3^-]$ is due in the majority of cases to excess of HPO_4^{--} and anions of undetermined acids. Only in two cases (24) (on both occasions) and (23) (on one occasion) does excess of Cl^- alone account for this diminution, and in four an absolute or relative excess of Cl^- together with an excess of HPO_4^{--} or undetermined anions. In four, (13), (32 *a*), (35) and (40), out of the six cases in which the $[HCO_3^-]$ was extremely low the reduction was wholly due to HPO_4^{--} and undetermined anions; of the other two, the reduction was mainly due to undetermined anions in case (9), while no conclusion is possible in case (39) as the analysis was not completed.

In five cases, (12), (13), (31), (32) and (40) the effect of the increase of these anions was modified by a reduction of $[Cl^-]$ both absolute and relative to

[Na^+]. In view of what has been said about the distribution of Cl^- it is not justifiable to conclude that there has been no retention of Cl^- in these cases, but in view of the high values for HPO_4^{--} and undetermined anions it can be said that there has been no specific retention of Cl^- in cases other than (24), and (23) on 28. vi. 22. Even in this last case on the second occasion, 14. vii. 22, there was an increase of undetermined anions and HPO_4^{--} in the plasma and none of Cl^-.

In all the cases the excess of HPO_4^{--} was less than the excess of undetermined anions or the excess of Cl^- relative to Na^+, so that the reduction of [HCO_3^-] is much greater than the excess of HPO_4^{--} would account for.

The CO_2 tension of the cerebrospinal fluid must be about the same as that of venous blood, so that in order to maintain the p_H of the fluid the same as that of plasma the [HCO_3^-] must be the same also. The average values found for the [HCO_3^-] in cerebrospinal fluid and plasma were about the same 0·0276 and 0·0280 g. equivalents per litre, and in the cases (25), (27), (32) and (33) in which the cerebrospinal fluid was examined at about the same time as the plasma the two agree closely. But in case (32) the low [HCO_3^-] in the cerebrospinal fluid is accounted for by excess of Cl^- instead of high HPO_4^{--} and undetermined anions, as in the plasma. Presumably, as in the process of secretion of cerebrospinal fluid the [HPO_4^{--}] and undetermined anions are kept low, the bicarbonate has to be reduced by a relative excess of Cl^-.

Of the patients with acidosis the only ones with dyspnoea were cases (23), with heart failure, and (37 *b*) (10. viii. 22). On the other hand, dyspnoea occurred in cases such as (22 *a*) (5. vii. 22) with high [HCO_3^-] and p_H. This dyspnoea therefore, as in the case of heart failure (52), was independent of the reaction and [HCO_3^-] of the plasma. Dyspnoea appears more as a manifestation of disturbance of the circulatory and respiratory apparatus than of acidosis. Moreover the patients with severe acidosis had little hyperpnoea. It was not noticeable in case (9) when she was sitting in the ward after coming up to hospital and it was not striking in the extreme cases (35 *a*) (16. xi. 22) and (13 *a*) (20. vii. 22). None of the cases had the extraordinary air hunger often seen in diabetic acidosis.

Although acidosis only occurred in severe cases it seemed, like urea retention, to cause little disturbance in itself, even when the p_H of the plasma was reduced. Case (13 *a*) (20. vii. 22) was quite clear-headed and case (35 *a*) (16. xi. 22) was only a little drowsy. Of the more severe cases of acidosis only case (40), who had a ketosis, was comatose; while case (9) was able to go about her work and case (24) (27. ii. 22) was able to get about.

Acidosis was not always present in uraemia, for example it was not present in cases (6), (11) and (29).

Table V.

Case		Plasma						Corpuscles	Blood			
		p_H	$[HCO_3^-]$	$[Cl^-]$	$[HPO_4^{--}]$	Sum	$[Na^+]$	$[Cl^-]$	$[Cl^-]$	Blood urea	Cell volume	
1	31. v. 22	—	0·030	0·088	(0·0035)	0·121	0·135	—	—	120	40	Acute nephritis.
	8. vi. 22	—	0·023	0·096	0·0025	0·121	0·126	—	—	54	40	Much improved.
2	1. xii. 21	—	0·030	0·102	(0·002)	0·134	—	—	—	25	—	Acute nephritis. Oedema.
	21. xii. 21	—	0·0285	0·1105	(0·002)	0·140	0·139	—	—	24	—	Much improved.
3	22. viii. 22	7·55	0·032	0·090	0·003	0·125	0·133	—	0·0735	141	33	Exacerbation of subacute nephritis. Slight oedema. Sodium bicarbonate given by mouth.
	6. ii. 22	—	0·0295	0·109	(0·0025)	0·141	0·146	—	—	75	40	Subacute nephritis; no oedema.
4	11. i. 22	—	0·026	0·102	(0·002)	0·130	0·130	—	—	38	—	,, ,,
5	6. ii. 22	—	0·030	0·106	(0·002)	0·138	0·144	—	—	19·5	50	Little change.
	3. vii. 22	7·4	0·027	0·0785	0·003	0·109	0·117	—	—	120	40	Infective endocarditis; uraemia; NaCl in urine 0·05 %.
6	13. vii. 22	7·3	0·030	0·102	0·002	0·134	0·1305	—	—	54·5	33	Free diuresis 5. vii. 22 to 7. vii. 22, great clinical improvement at same time.
	26. vii. 22	7·4	0·0265	0·1005	0·002	0·129	0·1305	—	—	31	33	Still better.
7	13. ix. 22	7·4	0·0275	0·1105	0·0025	0·140	0·142	—	—	43	25	Subacute nephritis; oedema. Plasma fibrin 0·31 %, globulin 1·53 %, albumin 2·15 %.
8	28. ii. 22	—	0·033	0·104	(0·002)	0·139	0·139	—	—	18	33	Subacute nephritis; oedema. Fits—probably hysterical.
9	25. i. 22	—	0·024	0·112	(0·0025)	0·138	0·142	—	—	96	50	Congenital cystic kidneys. Able to live moderately active life.
	8. iii. 22	—	0·025	0·114	0·002	0·141	0·142	—	—	102	40	— —
	26. x. 22	7·2	0·015	0·117	0·002	0·132	0·142	—	0·1006	141	33	No dyspnoea, nor hyperpnoea. Plasma fibrin 0·31 %, globulin 0·77 %, albumin 3·38 %.
10	25. i. 22	—	0·027	0·107	(0·002)	0·136	0·139	—	—	47	40	Subacute nephritis; no oedema. Serum Ca 0·010 %.
11	3. viii. 22	7·45	0·025	0·099	0·002	0·126	0·126	—	—	36	—	Pyelonephritis; one kidney removed 7 years ago. Occasional fits.
	10. x. 22	7·40	0·0265	0·101	0·003	0·130	0·133	—	0·0795	41	—	Plasma fibrin 0·31 %, globulin 1·12 %, albumin 2·16 %.
12	29. xii. 21	—	0·028	0·109	(0·003)	0·140	0·144	—	—	109	33	Pyonephrosis.
	12. i. 22	—	0·022	0·078	(0·006)	0·106	0·128	—	—	109	25	Much worse. Died 21. i. 22.
13	20. vii. 22	7·1	0·007	0·092	0·008	0·107	0·126	0·0565	—	320	—	Pyonephrosis; slight oedema. Fully conscious, no fits. Given 30 g. sodium bicarbonate in next 48 hours.
	22. vii. 22	7·2	0·013	0·088	0·009	0·110	0·126	—	—	320	16	Ca in serum 0·006 %. Fits started 8 hours later. Died next day.
14	2. iii. 22	—	0·034	0·102	—	0·136	0·135	—	—	22·5	—	Pyonephrosis.
15	24. viii. 22	7·5	0·029	0·096	0·002	—	0·126	—	0·0742	25	50	Mild pyelitis. Treated with sodium bicarbonate.
16	—	—	0·035	0·097	(0·002)	0·134	0·142	—	—	25	—	Chronic interstitial nephritis.
17	9. i. 22	—	0·030	0·102	(0·002)	0·134	0·128	—	—	41·5	—	Enlarged prostate. Fairly good condition.
	—	—	0·0335	0·096	(0·002)	0·131	0·130	—	—	37·5	—	— —

18	16. xii. 21	—	0·0265	0·1105	—	0·137	0·148	—	—	47	—	Enlarged prostate. Not considered fit for operation.
19	—. viii. 22	7·4	0·0295	0·099	0·002	0·1305	0·124	—	—	26	40	Chronic interstitial nephritis.
20	23. vi. 22	7·3	0·019	0·107	0·002	0·128	0·135	—	—	43	50	Chronic interstitial nephritis. Just recovering from fit, cyanosed.
	28. vii. 22	7·4	0·0285	0·104	0·003	0·135	0·130	—	—	40	40	Much better.
21	—	7·4	0·0245	0·109	0·002	0·135	0·133	—	—	63	30	Chronic interstitial nephritis. Heart failure. Dyspnoea.
22	5. vii. 22	7·45	0·0365	0·092	0·0025	—	—	—	—	58	40	Chronic interstitial nephritis. Moderate dyspnoea. Given 60 grains each of sodium bicarbonate and potassium citrate 3 times a day from 24. vi. 22.
	13. vii. 22	7·4	0·035	0·096	0·002	0·133	0·130	—	—	70	40	
23	28. vi. 22	7·35	0·022	0·113	0·002	0·137	0·135	—	—	83	40	Chronic interstitial nephritis. Heart failure, slight oedema. Dyspnoea.
	14. vii. 22	7·4	0·023	0·097	0·003	0·123	0·133	0·048	—	100	33	No oedema.
24	16. i. 22	—	0·018	0·1145	0·002	0·1345	0·135	—	—	126	40	Chronic interstitial nephritis. Ascites; no oedema.
Ascitic fluid	—	—	0·015	0·1215	0·002	0·1385	0·139	—	—	—	—	Globulin 0·3 %, albumin 1·2 %.
	27. ii. 22	—	0·014	0·1215	0·003	0·1385	0·139	—	—	120	40	Able to get about; no dyspnoea, slight hyperpnoea.
25	21. xii. 21	—	0·032	0·090	0·002	0·124	0·135	—	—	75	—	Chronic interstitial nephritis. Coma. No dyspnoea.
26	27. iv. 22	7·4	0·021	0·108	0·002	0·131	0·144	—	—	106	50	Chronic interstitial nephritis. Comatose. Serum Ca 0·010 %.
Spinal fluid	27. iv. 22	—	0·022	0·135	0·001	0·158	0·146	—	—	—	—	Given sodium bicarbonate 30 grains every two hours from 27. iv. 22 to 2. v. 22.
	2. v. 22	7·45	0·030	0·100	0·002	0·132	—	—	—	75	40	Much better.
	26. v. 22	—	0·022	0·100	—	—	—	—	—	92	50	Blood equilibrated with 6 % CO_2 before separation of plasma.
	23. vi. 22	7·3	0·025	0·101	0·0025	0·128	0·126	—	—	105	33	— —
	17. vii. 22	7·4	0·020	0·106	0·005	0·131	0·128	—	—	105	33	Coma and convulsions again; blood taken just after a convulsion.
27	9. x. 22	7·4	0·030	0·102	0·0015	0·133	0·130	0·060	—	50	—	Chronic interstitial nephritis.
Spinal fluid	—	—	0·032	0·124	0·001	0·157	0·142	—	—	—	—	— —
28	27. iv. 22	—	0·30	0·093	0·0025	0·125	0·148	—	—	120	25	Chronic interstitial nephritis. Drowsy; no dyspnoea.
29	31. vii. 21	—	0·031	0·082	(0·0075)	0·1205	0·135	—	—	152	—	Probably pyonephrosis. Unconscious; muscular twitching. Died 2. viii. 2.
30	16. vi. 21	—	0·0275	0·1105	(0·003)	0·140	—	—	—	131	—	Chronic interstitial nephritis.
	12. vii. 21	—	0·042	0·092	(0·004)	0·138	0·141	—	—	150	—	Given large doses of sodium bicarbonate from 7. vii. 21 to 14. vii. 21 then smaller doses.
	21. vii. 21	—	0·039	0·092	(0·0045)	0·135	—	—	—	138	—	— —
	2. viii. 21	—	0·0305	0·099	(0·004)	0·134	—	—	—	155	—	— —
	1. ix. 21	—	0·035	0·096	(0·0045)	0·135	0·139	—	—	172	—	Died 26. ix. 21 of bronchopneumonia.
31	21. viii. 22	7·3	0·0185	0·093	0·008	0·119	0·124	0·058	—	300	33	Chronic interstitial nephritis. No oedema, nor dyspnoea.

Table V *continued.*

Case		Plasma						Corpuscles	Blood			
		p_H	$[HCO_3^-]$	$[Cl^-]$	$[HPO_4^{--}]$	Sum	$[Na^+]$	$[Cl^-]$	$[Cl^-]$	Blood urea	Cell volume	
31	26. viii. 22	7·35	0·020	0·090	0·008	0·118	0·124	—	0·0735	480	30	Given 20 grains sodium bicarbonate and 10 grains potassium citrate 3 times a day throughout; sweated much in hot packs.
	30. viii. 22	7·35	0·0195	0·083	0·0080	0·110	0·124	—	0·0725	600	24	Plasma fibrin 0·53 %, globulin 2·41 %, albumin 3·62 %.
	4. ix. 22	7·4	0·021	0·085	0·007	0·113	0·137	—	0·0735	600	23	Appears better. Plasma fibrin 0·59 %, globulin 2·18 %, albumin 3·43 %.
	11. ix. 22	7·4	0·0165	0·077	0·011	0·104	0·128	—	0·0666	640	20	Worse, comatose. Plasma fibrin 0·61 %, globulin 1·37 %, albumin 2·72 %. Slight oedema of ankles at end. Died 13. ix. 22.
32	6. ix. 22	7·2	0·0125	0·096	0·011	0·119	0·133	0·0633	—	436	27	Chronic interstitial nephritis.
Spinal fluid	7. ix. 22	—	0·017	0·135	0·002	0·154	0·144	—	—	456	—	Given 30 grains acid sodium phosphate every 4 hours from 2. ix. 22 to 7. ix. 22, then large doses of sodium bicarbonate.
	8. ix. 22	7·4	0·016	0·087	0·01	0·115	0·131	—	0·077	564	27	Plasma fibrin 0·36 %, globulin 4·02 %, albumin 3·5 %. Possible loss of CO_2 in taking this blood. Urine NaCl 0·12 %.
	13. ix. 22	7·35	0·0185	0·074	0·018	0·110	0·142	0·050	—	600	20	Rapid shallow breathing. Plasma fibrin 0·51 %, globulin 2·78 %, albumin 2·75 %. Moderate oedema. Died 16. ix. 22.
33	8. vii. 22	7·4	0·027	0·096	0·003	0·126	0·130	—	—	57	—	Infective endocarditis. Moderate fibrosis of kidneys. Slight oedema. No dyspnoea.
	26. x. 22	7·6	0·0215	0·106	0·004	0·131	0·135	—	0·0923	171	25	Much worse; oedema; hyperpnoea. Plasma fibrin 0·50 %, globulin 0·49 %, albumin 3·39 %.
	31. x. 22	7·4	0·0245	0·104	0·0055	0·134	0·144	—	0·0915	240	23	Morphia given from 27. x. 22. Hyperpnoea much less; twitching.
Spinal fluid	6. xi. 22	—	0·0245	0·1455	0·002	0·172	0·165	—	—	—	—	Died 6. xi. 22. Taken 10 minutes after death.
34	31. x. 22	7·4	0·028	0·096	0·004	0·128	0·133	—	0·0725	171	40	Chronic interstitial nephritis. Had one fit and died 6. xii. 22.
35	16. xi. 22	7·2	0·006	0·1025	0·005	0·1135	0·135	—	0·098	343	20	Extensive fibrosis of kidneys. Conscious but drowsy. Ca in serum 0·010 %.
	20. xi. 22	7·55	0·0335	0·084	0·005	0·1225	0·139	—	0·0785	400	20	About 100 g. sodium bicarbonate on 18 and 19. xi. 22. Ca in serum 0·008 %. Died 8. xii. 22.
36	1. v. 22	7·4	0·020	0·116	(0·003)	0·139	0·146	—	—	68	33	Chronic parenchymatous nephritis. General oedema
37	6. vii. 22	7·4	0·0275	0·106	0·004	0·137	0·137	—	—	70	—	Chronic parenchymatous nephritis. General oedema.
	21. vii. 22	—	0·015	0·104	0·0035	0·122	0·119	—	—	71	—	Much worse; more oedema; dyspnoea.
	10. viii. 22	7·4	0·0195	0·094	0·0055	0·119	0·119	—	0·083	111	30	Condition unchanged.

38	12. v. 22	7·4	0·028	0·100	0·0033	0·131	—	—	—	115	—	Chronic parenchymatous nephritis. General oedema. Given 20 grains sodium bicarbonate 3 times a day from 24. v. 22.
	3. vii. 22	7·4	0·040	0·073	0·003	0·116	0·122	—	—	96	33	Much worse. More oedema. Serum Ca 0·008%.
	28. vii. 22	7·4	0·038	0·082	0·003	0·123	0·126	0·0735	—	96	25	Somewhat better.
39	21. vi. 22	7·2	0·0125	0·105	—	—	—	—	—	150	—	Only kidney removed for pyonephrosis 17. vi. 22.
	22. vi. 22	7·3	0·028	0·102	0·004	0·134	0·130	—	—	270	—	Given 20 grains sodium bicarbonate 3 times a day from 21. v. 22.
	26. vi. 22	7·35	0·029	—	0·003	—	—	—	—	330	—	— —
	29. vi. 22	7·35	0·028	0·066	0·002	0·096	0·117	0·047	—	450	—	Drowsy during last few days. Post mortem, slight oedema of back; pleural effusion 16 oz.
	P.M.	—	—	0·058	—	—	0·117	—	—	510	—	— —
40	31. vii. 22	7·25	0·014	0·082	0·002	0·098	0·119	0·0633	—	240	50	Diabetes, ketosis, necrosis of kidneys, anuria. Hyperpnoea slight, $NaHCO_3$ grains 20 T.D. from 29. vii. 22.
41	23. ii. 22	—	0·046	0·090	—	0·136	0·165	—	—	130	40	Toxaemia of pregnancy. Treated with enormous doses of sodium bicarbonate. Slow, shallow breathing, coma, tetany, death. Ca in serum 0·010 %.
Hyperpiesis:												
42	—	—	0·031	0·103	(0·002)	0·136	0·135	—	—	27·5	—	— —
43	5. xi. 21	—	0·033	0·102	(0·002)	0·137	0·133	—	—	33·5	—	— —
44	4. i. 22	—	0·036	0·104	0·003	0·137	0·137	—	—	26·5	—	— —
45	3. xii. 21	—	0·036	0·097	(0·002)	0·135	0·133	—	—	48	—	Given large doses of sodium bicarbonate.
	18. viii. 22	7·4	0·028	0·101	0·002	0·131	0·126	0·0666	—	54·5	50	No alkali given on this occasion.
46	27. vii. 22	7·45	0·030	0·095	0·003	0·128	0·126	—	—	39	50	— —
47	28. viii. 22	7·55	0·031	0·093	0·002	0·126	0·130	—	0·075	66	50	— —
Heart failure:												
48	14. viii. 22	7·4	0·0335	0·102	0·002	0·137	0·135	—	0·082	53	55	Much oedema, cyanosis and dyspnoea.
49	28. vi. 22	7·5	0·026	0·092	0·0015	0·119	0·122	—	—	43·5	55	Oedema, cyanosis and dyspnoea.
50	22. v. 22	7·35	0·0275	0·0965	0·0035	—	—	—	—	53	—	Oedema, cyanosis and dyspnoea.
51	9. iii. 22	7·5	0·029	0·1025	—	—	—	—	—	35	—	Little oedema, no cyanosis, great dyspnoea. Died of heart failure 25. xii. 22.
52	9. xi. 22	7·6	0·021	0·101	0·002	0·124	0·130	—	—	63	—	Infective endocarditis. Moderate oedema, cyanosis and dyspnoea.
	10. xi. 22	7·45	0·019	0·096	0·002	0·117	0·126	—	0·0885	56	30	Worse.
	11. xi. 22	7·45	0·020	—	—	—	—	—	—	54·5	—	Began to improve 12. xi. 22.
	15. xi. 22	7·45	0·0225	0·105	0·001	0·126	0·133	—	—	41	30	Much better, no dyspnoea.
	20. xi. 22	7·5	0·029	0·102	0·002	0·133	0·126	—	—	28	30	" "
No evidence of nephritis:												
53	—	7·4	0·0305	0·102	0·0015	0·134	0·133	—	—	—	—	Normal woman one day after delivery.
54	18. v. 22	7·35	0·027	0·098	—	0·125	0·126	—	—	24	—	Renal glycosuria. Serum Ca 0·010 %.
55	13. vii. 22	7·4	0·029	0·097	—	0·126	0·126	—	—	—	—	" "
56	8. viii. 22	7·5	0·024	0·110	0·002	0·136	0·135	—	0·093	28	—	Diabetes. No ketosis.
57	—	7·4	0·031	0·095	0·002	0·128	0·126	—	—	—	—	Normal adult.
58	—	7·4	0·0255	0·105	0·0015	0·132	0·133	—	—	—	—	"
59	—	—	0·0235	0·1105	0·0015	0·1355	0·135	—	—	—	—	Normal adult. Loss of CO_2 in collecting blood.

Summary and Conclusions.

1. $[HCO_3^-]$, $[Cl^-]$, $[HPO_4^{--}]$ and $[Na^+]$ were determined in plasmata and in a few cerebrospinal fluids of patients with nephritis, also in plasmata and cerebrospinal fluids of control cases.

2. The plasma $[HPO_4^{--}]$ was increased in the majority of cases with high blood urea.

3. $[Na^+]$ was not increased in the plasma even in extreme degree of renal inefficiency, on the other hand low values were found in several such cases.

4. Evidence is given of disturbance of the distribution of chlorides between the plasma on the one hand and cerebrospinal fluid and tissues on the other.

5. Very low figures for plasma $[Cl^-]$ were found in some severe cases of nephritis.

6. The excess of kation in normal plasma unaccounted for by $[HCO_3^-]$, $[Cl^-]$, and $[HPO_4^{--}]$ is combined mainly with protein; a little may be combined with organic anions.

7. In many cases of nephritis with much urea retention an excess of kation over and above that combined with HCO_3^-, Cl^-, HPO_4^{--} and protein was found. This was combined with anions, sulphanion among others, classed as undetermined anions. Such undetermined anions were found in cases with and without acidosis.

8. The p_H of the majority of cases lay within normal limits, especially about 7·40. High values up to 7·6 were found, these were caused by disturbances of the respiratory centre and by treatment with sodium bicarbonate.

9. In most cases of acidosis in nephritis, the reduction of plasma $[HCO_3^-]$ was due to accumulated HPO_4^{--} and undetermined anions; of these, undetermined anions were the more important factor. In several cases the effect of these anions was much reduced by a coincident fall of $[Cl^-]$. In only one case was there evidence of a specific retention of Cl^- as the cause of the acidosis.

10. Dyspnoea in nephritis is not caused by acidosis but by disturbances of the circulation and respiration. Hyperpnoea in acidosis in nephritis is not striking even in severe cases.

11. Acidosis in nephritis is evidence of a very severe condition but is not harmful in itself. It does not cause any of the symptoms of uraemia.

12. The $[HCO_3^-]$ of the cerebrospinal fluid is the same as that of the plasma. Reduction of $[HCO_3^-]$ in cerebrospinal fluid is caused by excess of $[Cl^-]$.

I take this opportunity to thank the Physicians of the London Hospital for the facilities offered me in the investigation of these cases; also my wife, Miss D. S. Russell and Mr T. Cathrall for the estimation of phenolsulphonephthalein excretion, of urea in blood and urine, and other assistance.

REFERENCES.

Austin, Cullen, Hastings, McLean, Peters and Van Slyke (1922). *J. Biol. Chem.* **54**, 144.
Bell and Doisy (1920). *J. Biol. Chem.* **44**, 55.
Berglund (1922). *J. Amer. Med. Ass.* **79**, 1375.
Briggs (1922). *J. Biol. Chem.* **53**, 13.
Clark (1921). *J. Biol. Chem.* **44**, 487.
Cullen (1922). *J. Biol. Chem.* **52**, 501.
Denis (1921). *J. Biol. Chem.* **49**, 311.
—— and Meysenbug (1922). *J. Biol. Chem.* **52**, 1.
—— and Minot (1920). *Arch. Int. Med.* **26**, 99.
Doisy, Eaton and Chouke (1922). *J. Biol. Chem.* **53**, 61.
Fraser, Ross and Dreyer (1922). *Quart. J. Med.* **59**, 193.
Greenwald and Lewman (1922). *J. Biol. Chem.* **54**, 263.
Halverson and Bergeim (1917). *J. Biol. Chem.* **29**, 337.
Kramer (1920). *J. Biol. Chem.* **41**, 263.
—— and Tisdall (1921). *J. Biol. Chem.* **44**, 467.
—— —— (1922). *J. Biol. Chem.* **53**, 241.
Myers (1917). *J. Biol. Chem.* **29**, 337.
—— and Short (1921). *J. Biol. Chem.* **48**, 83.
Norgaard and Gram (1921). *J. Biol. Chem.* **49**, 263.
Peters, Barr and Rule (1921). *J. Biol. Chem.* **45**, 571.
Van Slyke (1922). *J. Biol. Chem.* **53**, 495.
Van Slyke, Stillman and Cullen (1919). *J. Biol. Chem.* **38**, 167.
Wahlgren (1909). *Arch. exp. Path. Pharm.* **61**, 97.
Wu (1922, 1). *J. Biol. Chem.* **51**, 21.
—— (1922, 2). *J. Biol. Chem.* **51**, 33.

XXXI. RATE OF LIBERATION OF ACID BY $\beta\beta'$-DICHLORODIETHYL SULPHIDE AND ITS ANALOGUES IN ITS RELATION TO THE "ACID" THEORY OF SKIN VESICATION.

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Introduction.

The theory that the intracellular liberation of acid is the effective agent in producing vesication by $\beta\beta'$-dichlorodiethyl sulphide (described as DES throughout this report) has received support by E. K. Marshall [1919, 1920] and other American workers [Lynch, Smith and Marshall, 1918], though others [Warthin and Weller, 1919] oppose this view. Two ideas seem to be involved, (*a*) that owing to its ready lipoid solubility, DES easily reaches the inside of the cell, and (*b*) that in the cell, DES splits off acid at a favourable rate for the production of pathological change. If the theory is correct, analogous lipoid-soluble substances which split off acid either at the same rate or faster than DES should be vesicant, and conversely analogous substances, which split off acid much slower than DES should be either non-vesicant or very much less vesicant than DES. The test of the theory therefore depends upon an investigation of the rate at which acid is split off from DES and analogous compounds under conditions approximating as nearly as possible to the conditions under which DES would break down in the body cell.

Previous work upon the rate of acid liberation by DES has been published. Rona [1921] investigated by a conductivity method the rate of so-called hydrolysis of a series of compounds, including DES and dichlorodimethyl sulphide. His general conclusions were that dichloromethyl sulphide hydrolysed "quickly, but measurably," DES "gradually," tetrachlorodiethyl sulphide "gradually"; DES hydrolysed according to the monomolecular law.

Russell [Report] by the same method concluded that the reaction proceeded in two stages:

$$(1)\quad S\begin{<}CH_2CH_2Cl\\CH_2CH_2Cl\end{>} + H\cdot OH = S\begin{<}CH_2CH_2OH\\CH_2CH_2Cl\end{>} + HCl$$

$$(2)\quad S\begin{<}CH_2CH_2OH\\CH_2CH_2Cl\end{>} + H\cdot OH = S\begin{<}CH_2CH_2OH\\CH_2CH_2OH\end{>} + HCl$$

though he found difficulty in interpreting some of the data obtained. Other workers have employed titration methods. E. F. Hopkins [1919] concluded

that the rate of liberation of acid proceeded according to the monomolecular law. The method used by him, viz. of withdrawing samples and cooling in mixtures of ice and calcium chloride, gave unreliable results in our hands owing to the difficulty of stopping the reaction during the cooling.

There is evidently some disagreement as to whether the reaction follows the monomolecular law. Some workers have found difficulty in accepting the "acid" theory. Flury and Wieland [1921] for instance suggest in a discussion that oxidation in the cell may play its part in the formation of vesicant substances from DES. In support of this they quote dichlorodiethyl sulphoxide and dichlorodiethyl sulphone, both of which they consider to be vesicant[1]. The suggestion that substances formed by the cell from DES may be the actual vesicants is of some importance, if the "acid" theory cannot be accepted. We have investigated the rate at which several of these substances yield acid by the following methods.

METHODS.

Whatever method is adopted, the first point to be settled is that of obtaining a satisfactory solution. Hopkins [1919] by vigorously shaking DES with large quantities of water and subsequently withdrawing the clear supernatant solution, estimated the solubility of DES in water to be of the order of 0·07 % at 10°. Boulin and Simon [1920] obtained a figure of 0·048 %. The solubility is therefore slight. Experimentally we found that when 0·04 % of DES was shaken with water the whole body of the solution was filled initially with minute droplets. An examination of the solution under the microscope even after some minutes, by refracted light, showed the presence of finely suspended droplets, after the solution had become apparently clear to the naked eye. A microscopic examination of two solutions made (1) by shaking DES alone with water, and (2) by adding an alcoholic solution of 0·04 % DES to water made it clear that the alcoholic solution became homogeneous much more rapidly. Accordingly we have used for our experiments solutions of DES made by adding water to DES dissolved in alcohol, the total strength of DES being not more than 0·04 %, and of alcohol usually not more than 5 %. Freshly made, these solutions show a turbidity, which clears upon shaking in the course of a minute.

In the choice of a method of estimating the amount of acid liberated, we have been guided by two considerations, firstly that the acid should be estimated as such, and secondly that the acid should be liberated under conditions approximating to those in the living cell. Recent research has shown that the organism is able to call upon large reserves of alkali when necessary, in fact the reaction of the blood is adjusted with great accuracy in the region of p_H 7·4 under wide variation of external condition. This seems to indicate that in the cell the liberation of acid by DES must be accompanied by a neutralisation of the acid formed, at any rate in the initial stages. The body

[1] Marshall considers the "sulphone" to be vesicant, but not the "sulphoxide."

cell will carry out a titration of the acid at constant reaction. In the case of the body cell the extreme limits of reaction will not be greater than p_H 6·0–8·0. The idea of withdrawing samples from a DES solution in which the concentration of hydrogen ions was gradually accumulating was therefore abandoned. Instead, the progress of the reaction was estimated by titrating to a constant, slightly acid reaction. Most experiments were done using methyl-red as indicator, with an end point of p_H 5·3, using CO_2-free alcoholic soda (N/100) as titration solution. By the use of methyl red, errors introduced by the taking up of CO_2 during the experiments were largely avoided. The additions of alkali were kept low (2 cc. of N/100 NaOH at a time), so that without the use of buffer solutions the liberation of acid could be studied within the limits of reaction p_H 5·0–9·0 approximately. A final control in the presence of M/1000 phosphate in which the reaction could be kept accurately between the limits p_H 7·0–7·4 gave essentially the same course for the acid curves. The rate of acid liberation is obtained complicated only by a slight dilution of the solutions with water and by a gradual accumulation of traces of NaCl in the solution. These factors will be discussed below.

Description of method.

The method finally adopted was as follows. Distilled water, subsequently boiled to drive off CO_2 and cooled with a soda lime tube attached to the containing flask, was used throughout. Two flasks, one containing 95 cc. of CO_2-free water plus indicator (methyl red) and the other, 5 cc. of absolute alcohol, were placed in the bath and allowed to assume the required temperature. Two drops of DES[1] (0·0384 g.) were then transferred by means of a dry pipette to the flask containing the alcohol. Next the 95 cc. water were poured rapidly into the alcoholic solution, and the stop watch started, the receiving flask being shaken whilst immersed in the water. A clear solution was obtained in about half a minute. After the lapse of a minute (to ensure complete solution) the titration was started, using 5 % alcoholic soda, N/100. Alkali was added until the solution, at first pink, went yellow. The burette was then read. The time of return of the colour to pink as compared with a standard flask clamped in the bath was then noted. The water-bath was of white enamel in order to aid the observation of these colour changes. As soon as the time of return was noted, more alkali (1–2 cc.) was added and the observations repeated. This was continued until it was judged by the slowness of the change that the stage of complete hydrolysis was nearly reached. During the whole of these operations, the flask was kept immersed in the water and subjected to gentle shaking. It was possible to observe the return of colour with great accuracy, the time readings being correct to the second. When the reaction was apparently finished, the solution was diluted with distilled CO_2-free water and heated upon the water-bath. Upon becoming acid more alkali

[1] The DES used was made by the sulphur monochloride method and purified by fractional distillation under reduced pressure.

was added until neutral, this being continued until no more acid was formed, usually 24 hours. When not on the water-bath the flasks were kept tightly stoppered to prevent access of CO_2. The above method was subject to such variations as the experiments demanded.

The determination of the end point, that is the maximum amount of acid which could be liberated, gave a certain trouble. Other workers have used Volhard's method for the total chloride. This was tried, and also Larrson's modification of Volhard's method, but both were rejected owing to the indefiniteness of the end point. This may be due to the interference of thiodiglycol in silver titrations discussed by the French workers [Moureu and Murat, 1920]. The method described above was finally adopted as giving the most satisfactory results. It must be noted that under these conditions there is no strict equilibrium point. The DES splits down smoothly, until all of it has disappeared. This was judged both from the titration values and also by skin tests. A number of solutions, in which hydrolysis was considered to be complete by titration values, were tested on the skin of the forearm for blistering power. In all cases a negative result was obtained.

The validity of the method.

In the case of DES we are not dealing with a case of saponification by alkali of the ordinary type. Liberation of HCl by the action of water proceeds at a markedly acid reaction in the absence of alkali. It is only slightly inhibited in the presence of $N/100$ HCl (at a $p_H = 2{\cdot}0$). The addition of NaOH therefore does not initiate this reaction. Hence in a justification of the method, we are concerned with showing

(*a*) what is the effect upon the course of the reaction of the addition of small amounts of alkali;
(*b*) what is the effect of allowing the reaction to proceed in the presence of accumulating acid;
(*c*) what is the influence of the indicator, if any;
(*d*) what disturbance is introduced by the presence of accumulating NaCl.

The effect of dilution can be left out of account as the solution initially contains 2000 times more water than DES.

(*a*) *The effect of alkali.*

Fig. 1 is a curve made from three separate experiments, in which the percentage acid liberated is plotted against the time. It will be seen that the observations lie quite smoothly; in itself this constitutes indirect evidence that the addition of alkali has not appreciably disturbed the course of the reaction, because it is impossible to add accurately the same amounts of alkali at each stage, owing to the speed at which the reaction is proceeding. We tried to find any accelerating effect of alkali by carrying out the experiments in the presence of 15–20 cc. of alkali added initially. Points marked "alkali"

points in Fig. 1 were obtained in this way. It will be seen that the presence of 5–10 times as much alkali as is normally present throughout a considerable part of the reaction only leads to small increases in the amount of acid liberated. The largest increase amounts to some 3 %. This makes it evident that upon the alkaline side of the neutral point the rate of acid liberation is remarkably independent of p_H. The p_H in these experiments must have been more alkaline than p_H 11·0 during part of the reaction.

The details of the experiment are shown in Experiment 1.

Exp. 1. A 300 cc. solution was made up as previously described. Immediately after mixing, portions of 100 cc. measured roughly were decanted into two flasks, the first containing 15 cc. *N*/100 NaOH and the second 20 cc. *N*/100 NaOH. The original flask in which about 100 cc. solution remained was titrated in the standard manner. The two flasks containing alkali initially were kept under observation and the time of return of colour from yellow to pink noted. All three flasks were kept immersed in the same water-bath throughout.

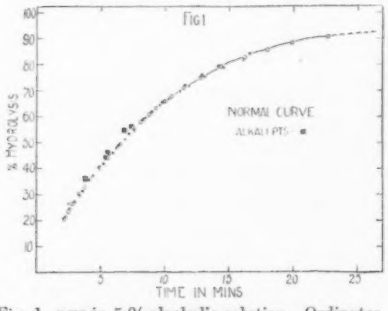


Fig. 1. DES in 5 % alcoholic solution. Ordinates % acid liberated. Abscissae Time. Temp. 24·5±0·10. Points ● ⊙ + represent three different experiments. Points marked ■ obtained in the presence of alkali.

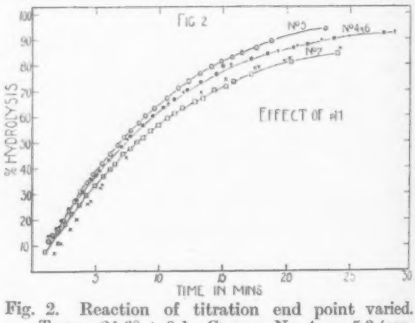


Fig. 2. Reaction of titration end point varied. Temp. 24·6° ± 0·1. Curves: No. 4, p_H 5·3 (control). No. 5, p_H 9·0. No. 6, p_H 7·1. No. 7, p_H 9·0 exposed specimen of DES. *X*, p_H 7·0 (in presence of phosphate *M*/1000).

Confirmation of this unexpected behaviour to alkali was obtained by performing titrations to end points of varying reaction, using indicators other than methyl-red. In the case of the experiments with thymol-blue (end-point p_H 9·0), especial care was taken to keep the reaction alkaline throughout, the fresh alkali being added at the instant of the return of colour. Fig. 2 shows the curves obtained. Nos. 4 and 6 were at p_H 5·3 and 7·1 respectively. Nos. 5 and 7 were both at p_H 9·0, No. 5 is slightly accelerated as compared with Nos. 4 and 6. No. 7 shows the greatest divergence from its fellow No. 5. It is included here in order to introduce a puzzling irregularity which we have encountered, in specimens of DES which have been repeatedly exposed to the air. We have found a tendency to a slowed rate of acid liberation in such specimens, which cannot be accounted for by the supposition of preformed acid.

The points marked *X* in Fig. 2 were obtained at a reaction p_H 7·0–7·4 by

using traces of phosphate as buffer (M/1000). It is evident that there is no significant difference from the other curves.

(*b*) *The effect of acid.*

Fig. 3 shows the effect upon the course of the reaction of (*a*) added acid and (*b*) of the maintenance of an acid reaction of p_H 3·6 approximately. The details of the experiment were as follows:

Exp. 2. 400 cc. solution were made, containing the usual 0·04 % DES and 5 % alcohol. Immediately upon solution, portions of about 100 cc. were rapidly decanted into three flasks, two of which contained 15 and 20 cc. of N/100 HCl respectively. All four flasks were left in the water-bath. At intervals the flasks were titrated. The end points were obtained in the usual way, allowing for the presence of the added acid where necessary.

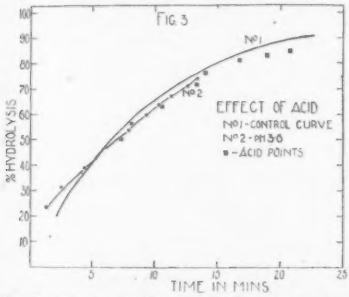


Fig. 3. Effect of acid. Ordinates and abscissae as before. Curve 1. Temp. 24·5°±0·1°. Curve 2. Temp. 24·6°.

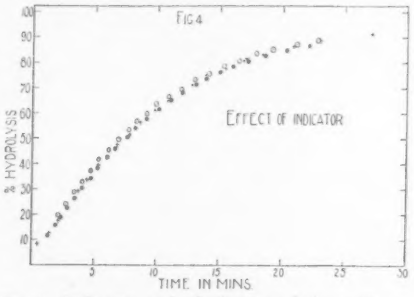


Fig. 4. Ordinates and abscissae as before. Temp. 24·6°±0·1. Effect of indicator and small increase in alcohol. 1. Normal curve from Fig. 1, ●. 2. Twice normal amount of indicator, ⊙. 3. Normal amount of indicator plus 6 % alcohol, +.

The points obtained from the flasks containing initial acid are seen to lie well off the control curve. This slowing effect of acid has been noted by other observers. It is brought out in the experiment conducted at a reaction of p_H 3·6, with bromphenol blue as indicator, though the latter experiment was not satisfactory owing to the difficulty of obtaining readings of the colour change. Under the conditions of the method, where the flasks are only allowed to go acid momentarily, this slowing effect of acid is avoided as a complication of the results.

(*c*) *The effect of indicator.*

With one or two exceptions the indicator used throughout was methyl-red. This indicator is made up in 60 % alcoholic solution and the amount used in any experiment was of the order of 0·7 %, containing 0·00014 g. methyl-red. As no allowance was made for the extra alcohol added in this way, the effect of doubling the amount of indicator by weight was tried, as also that of increasing the strength of alcohol to 6 % instead of 5 %. The results are shown diagrammatically in Fig. 4. We may conclude that the indicator added had no effect upon the course of the reaction.

(d) Effect of NaCl *formed during the progress of the reaction.*

There is a slight effect produced by the accumulating NaCl which will be discussed later. In the early stage of the reaction, up to 60 % hydrolysis[1], the effect is slight.

From the above it will be seen that the precautions to be adopted in using the method are (1) to add the alkali in small amounts at a time and (2) not to allow the solutions to remain acid at any stage.

We have compared by this method the rate of liberation of acid from DES in water solutions only, in mixtures of water and alcohol, at various temperatures, in the presence of salts, and in the presence of a few substances of physiological interest.

THE COURSE OF THE REACTION.

Table I is the record of a typical experiment in 5 % alcohol; k is calculated from the equation

$$k = \frac{1}{t_1 - t_0} \log_e \frac{a - x_0}{a - x_1},$$

t refers to time and x to % acid liberated. t_0 and x_0 are taken at a convenient point between 1 and 2 minutes after the beginning of the reaction, in order to avoid the influence of irregularities at the start, or of traces of preformed acid in the specimen of DES.

Table I. *Temp.* 24·6°.

Time	% A	k	Time	% A	k
1′ 18″	12·6	—	10′ 19″	63·7	·099
1′ 54″	16·7	—	11′ 10″	66·8	·100
2′ 19″	24·3	·094	12′ 14″	70·3	·100
3′ 30″	27·9	·090	13′ 20″	73·4	·100
3′ 57″	31·0	·092	14′ 30″	76·6	·101
4′ 43″	35·5	·091	14′ 59″	79·7	·109
5′ 15″	39·1	·094	17′ 10″	82·1	·101
6′ 2″	43·3	·094	18′ 30″	84·2	·101
6′ 57″	48·5	·096	20′ 30″	86·8	·099
7′ 43″	52·5	·097	21′ 35″	87·9	·098
8′ 41″	56·7	·097	23′ 42″	90·0	·097
9′ 29″	60·4	·099	27′ 40″	92·4	·093

Here, and throughout, A refers to the amount (percentage) of acid liberated at the time stated and is calculated upon the basis of total amount of acid liberated as determined by the final titration.

k calculated upon a monomolecular basis, increases slightly during the stage 28–82 % hydrolysis and then falls to 90 % hydrolysis, the total variation being 0·090 to 0·101, that is about 11 %. In general it may be said that the reaction simulates a monomolecular reaction under these conditions, although over no range is k absolutely constant. There is a steady rise to 80 % hydrolysis, a rise which is masked perhaps slightly by the presence of accumulating NaCl in the solution. The extent of this effect will be considered later. For con-

[1] The term "hydrolysis" may not be strictly correct. It is used for convenience to represent "liberation of acid."

venience of comparison, the reactions can be compared by interpolating from the curves the values of k at 30, 40, 50, 60, 70, 80 % acid liberated. Table II gives the results of a series of experiments with 0·04 % DES in the presence of 5 % alcohol.

Table II.

	k						
Exps.	30% A	40% A	50% A	60% A	70% A	80% A	Remarks
1	·093	·096	·100	·102	·102	·102	No phosphate. Reaction p_H 5·0–9·0. Temp. 24·6±·1°
2	·091	·093	·094	·096	·098	·098	,, ,,
3	·091	·093	·095	·097	·098	·098	,, ,,
4	·090	·092	·094	·096	·097	·096	,, ,,
5	·093	·095	·100	·100	·100	·099	No phosphate. Indicator twice the normal amount
6	·092	·094	·095	·097	·098	·098	No phosphate. 6 % alcohol instead of 5 %
7	·090	·093	·094	·095	·094	·092	Phosphate M/1000. p_H 7·2–7·4
8	·084	·088	·093	·094	·095	·090	,, ,,

The reaction seems to be most stable at 50–60 % hydrolysis, and we may take the mean of the 50 and 60 % constants as a characteristic constant χ of any particular reaction. In Experiments 1–6 this becomes 0·097, for experiments 7 and 8 0·094, slightly lower.

The effect of different conditions upon the course of the reaction may be judged (1) from changes in the characteristic constant, and (2) from changes in the curve relating the value of the constant χ and the % acid liberated.

Water only.

There is no appreciable difference between Exp. 1, Table III, with three drops of DES % and Exp. 2 with one drop of DES %; this shows that within these limits, variations in concentration of DES do not affect the % liberation of acid in a given time. $\chi = 0{\cdot}105$, *i.e.* some 8 % higher than in the case of 5 % alcohol. It is difficult to decide whether this is actually a slowing effect of the alcohol or whether it is due to some solubility question. As t_0 and x_0 were taken some time after the beginning of the reaction, the irregularity due to slow solution should be excluded.

Water and alcohol.

Fig. 5 and Table III, Exps. 5, 6, 7, 8 give the results. Alcohol slows the reaction, χ for 25 % = ·090 and for 50 %, χ = ·0163. The constant at 25 % behaves as that of a true monomolecular reaction throughout the range, whereas with 50 % alcohol k increases 60 % between 30 and 80 % hydrolysis. This shows that alcohol is also affecting the course of the reaction.

Temperature.

Fig. 6 and Table III, Exps. 6, 9, 10 show the results. The characteristic constant χ is ·028 at 14·5°, χ = ·097 at 24·6° and ·385 at 36·8°. This makes the temperature coefficient between 14·5–24·7 = 3·34, and between 24·7 % and 37·0 % = 3·22, the average being 3·28 per 10° rise in temperature. It is to be noted that at 37° the reaction behaved almost strictly throughout as a monomolecular reaction.

Table III. *Course of reaction under varying conditions.*

Group	Exps.	k 30 % *A*	40 % *A*	50 % *A*	60 % *A*	70 % *A*	80 % *A*	Alcohol %	Remarks
Water	1	—	·161	·103	·106	·107	·108	nil	3 drops DES %
	2	·089	·101	·104	·106	·106	·104	,,	1 drop DES %
	3	—	·107	·105	·107	·108	·108	,,	2 drops DES %
	4	·029	·030	·031	·031	·031	·031	,,	Temp. 14·7°
Alcohol	5	·093	·097	·101	·103	·107	·112	1	Temp. 24·8°±·4° (Fig. 5, No. 2)
	6	·091	·093	·094	·096	·098	·098	5	— —
	7	·090	·0895	·089	·0905	·091	·091	25	Temp. 24·7° (Fig. 5, No. 4)
	8	·013	—	·016	·017	·018	·019	50	Temp. 24·4°
Temperature	9	·027	·028	·028	·029	·030	·031	5	Temp. 14·5°
	10	—	—	·38	·39	·385	·39	,,	Temp. 36·8°
NaCl	11	·095	·091	·094	·095	·095	·093	,,	·01 % NaCl in *M*/1000 phosphate
	12*	·088	·083	·083	·086	·083	·080	,,	·05 % in *M*/1000 phosphate
	13	·066	·069	·070	·071	·072	·072	,,	Temp. 24·4°. 0·1 % in *M*/1000 phosphate
	14	·028	·029	·031	·032	·033	·033	,,	Temp. 24·5°. 1 % in *M*/1000 phosphate
	15	·010	·011	·012	·012	—	—	,,	Temp. 24·7°. 5 % in *M*/1000 phosphate
Other salts	16	·077	·077	·077	·075	·070	·038 (83 %)	,,	Temp. 25·4°. 1 % Na_2SO_4
	17	·063	·062	·059	·051	·038 (68 %)	—	,,	Temp. 24·8°. 1 % $MgSO_4$
	18	·094	·095	·097	·098	·098	·098	,,	Temp. 24·5°. 1 % $NaNO_3$ in *M*/1000 phosphate
Other substances	19	·077	·078	·080	·082	·082	·081	,,	·1 % diethylamine
	20	·089	·090	·094	·096	·097	·096	,,	·036 % lecithin
	21	—	·081	—	·087	·088	·090	,,	·1 % glycine hydrochloride
	22	·026	·027	·029	·030	·031	·031	,,	Ringer's solution instead of water

The temperature can be relied upon to ±0·1°. Temp. 24·6° unless otherwise stated.

* The controls to this experiment are Table II, Exps. 7 and 8.

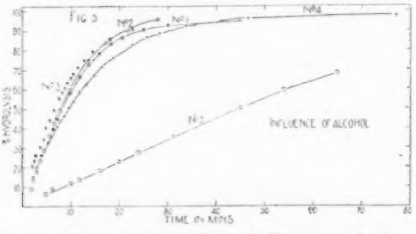


Fig. 5. Influence of alcohol. Ordinates and abscissae as before. Temp. 24·5°±0·1°. Curves: No. 1, no alcohol ●. No. 2, 1 % alcohol ⊙. No. 3, 5 % alcohol ✻. No. 4, 24 % alcohol +. No. 5, 50 % alcohol □.

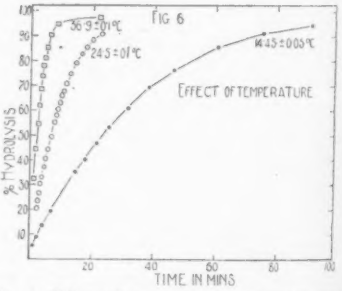


Fig. 6. Effect of temperature. Ordinates and abscissae as before.

Salts.

Fig. 7 and Table III, Exps. 11–18 inclusive show the results. 1 % NaCl changes χ at 24·6° from ·097 to ·028, practically the equivalent of 10° fall in temperature. 0·1 % NaCl in the presence of phosphate, reduced χ from ·094 to ·070, a reduction of 20 %. It is to be noted that the course of the

reaction is not changed, there is still the gradual increase in k, which is found in the absence of added NaCl.

The effect of sulphates is remarkable, up to 50 % hydrolysis very little change is noted in the course of the reaction, but after 50 % the reaction slows rather abruptly, and proceeds to completion only after the lapse of several days. We have not attempted to investigate the cause of this remarkable effect of sulphate.

Sodium nitrate (1 %), it will be seen, has no effect upon the reaction.

Some other substances.

Table III, Exps. 19, 20, 21, 22 show the effect of diethylamine, lecithin, glycine hydrochloride and Ringer's solution. The slight retardation observed with glycine and diethylamine is undoubtedly due to the effect of chlorides. Ringer's solution has the effect which can be expected from its salt content.

Summary of Results.

Summarising the above data, the reaction from the broad standpoint resembles a monomolecular reaction. It is apparent however that we may be dealing with a balance of factors, masking a more complicated reaction for the following reasons:

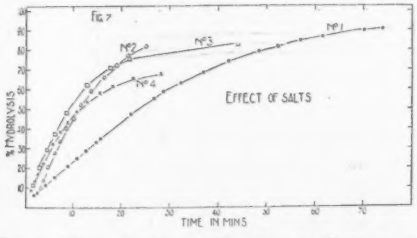


Fig. 7. Effect of salts. Ordinates and abscissae as before. Curves: No. 1, 1 % NaCl. No. 2, 0·1 % NaCl (in presence of $M/1000$ phosphate). The controls to this curve are given in Fig. 2. No. 3, 1 % Na_2SO_4. No. 4, 1 % $MgSO_4$. Temp. Nos. 1, 2, 4, 24·5° ±0·1°. No. 3, 25·4°.

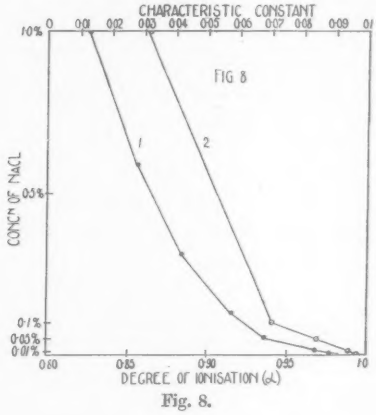


Fig. 8.

(1) There is usually a definite increase in the constant k as the reaction proceeds from 30–80 % hydrolysis. This increase found under the conditions of experiment used is smaller than would be observed in the absence of accumulating NaCl. If we correct the data of Exp. 2, Table II by extrapolation for the amount of NaCl retardation at different stages from the known data from Exps. 11–15, we obtain the following figures, Table IV.

Table IV.

	k					
	30 % *A*	40 % *A*	50 % *A*	60 % *A*	70 % *A*	80 % *A*
Uncorrected	·091	·093	·094	·096	·098	·098
Corrected	·093	·096	·098	·100	·103	·104

It is not pretended that these corrected figures are more than an approximation to the truth, but they tend to confirm the opinion that the action of water upon DES is not absolutely simple.

(2) The course of the reaction is not always the same quite apart from any slowing effect. Sometimes as in the case of 25 % alcohol at 24·6°, and with 5 % alcohol at 37·0° the reaction behaved almost strictly as a monomolecular reaction. With 50 % alcohol, there is a much more rapid acceleration than normal as the reaction proceeds. With sodium sulphate the reaction is completely changed. This would receive a simple explanation if the reaction went in two stages, one of which was inhibited by sulphate. The subject will be discussed below.

Meanwhile one generalisation seems clear. Conditions which influence the reaction all slow the rate at which DES yields acid. In the cell therefore in which we have a concentration of some 0·5 % NaCl, the rate of liberation of acid will be slow. It will be further slowed by the removal of DES from the aqueous part of the cell by solution in lipoid phases. In itself this militates against the "acid" theory of vesication.

The next section deals with the behaviour of analogous compounds.

ANALOGUES OF DES.

The following were placed at our disposal: $\alpha\alpha'$-dichlorodimethyl sulphide, $\alpha\beta\beta'$-trichlorodiethyl sulphide, $\alpha\beta\beta\beta'$-tetrachlorodiethyl sulphide and $\alpha\alpha\beta\beta\beta\beta'$-hexachlorodiethyl sulphide. Of these the first named liberated acid very readily in contact with water, and the remainder only with difficulty. In the case of dichlorodimethyl sulphide, a clear homogeneous solution was obtained under the same conditions as with DES. Table V gives the details of three experiments.

Table V. *Dichlorodimethyl sulphide.*

	k						
Exps.	30 % *A*	40 % *A*	50 % *A*	60 % *A*	70 % *A*	80 % *A*	Remarks
1	·16	·16	·15	·15	·15	·15	Temp. 14·7°
2	—	·42	—	·40	·40	·40	Temp. 24·6°. *M*/1000 phosphate
3	—	·47	—	·47	—	·44	Temp. 24·6°. *M*/1000 phosphate, NaCl 0′ %

Acid is liberated from dichlorodimethyl sulphide at a markedly faster rate at 14·7° than from DES at 24·6°, the velocity constant at the same temperature being some five times greater. Further it must be noted that the presence of 1 % NaCl does not slow this reaction, thereby indicating a difference of chemical behaviour between the two compounds.

There is another respect in which the compound differs from DES. During

the progress of the reaction, a fine white precipitate[1] gradually accumulates; this is not sufficient to interfere with the colour observations. The end point is reached almost immediately after the observations are discontinued, there is no need to heat or to allow the mixture to stand for some time. The insolubility of the precipitate probably removes the hydrolytic product from the sphere of action, so allowing the reaction to go quickly to completion. This is concordant with the steady fall observed in the constant.

With regard to the other three compounds, it is difficult to get reliable data. They were all much less soluble in water than DES. 5 % alcohol failed to take them into solution. 25 % did not ensure complete solution except in the case of the trichloro derivative. It seemed essential to obtain a homogeneous solution before any measurements were attempted, therefore 50 % alcoholic solutions were used. Under these conditions complete solution was assured, but even at 38° liberation of acid was slow. Relative measurements were obtained finally at 52°. These indicated that under the prescribed conditions, acid is liberated most readily from the hexachloro compound (50 % in 70 minutes), and least readily from the trichloro compound (no apparent hydrolysis). The tetrachloro compound occupies an intermediate position (50 % hydrolysis in 100 minutes). Under the same conditions (50 % alcohol solution), calculating from the results at 25°, DES should liberate 50 % of its acid in three minutes. k for the hexachloro derivative is shown in the following table, it falls throughout the reaction.

k is here calculated according to the equation

$$k = \frac{1}{t} \log_e \frac{a}{a-x}.$$

Table VI. *Hexachlorodiethyl sulphide.*

% A	k	% A	k
4·5	·021	34·3	·016
8·6	·021	38·4	·015
13·8	·022	42·6	·013
19·6	·023	45·7	·011
24·1	·021	49·0	·010
29·2	·020		

Dichlorodiethyl sulphone.

The following experiments, Table VII, show that dichlorodiethyl sulphone hydrolyses about 100 times less rapidly than DES at 37°, and that NaCl has a depressant action upon it similar to that upon DES.

Table VII.

Exp. 1. Temp. 37·0°			Exp. 2. Temp. 37·2° 1 % NaCl			Exp. 3. Temp. 24·6° 1 % NaCl		
Time	% A	k	Time	% A	k	Time	% A	k
34′	10·7	—	40′	5·1	·0013	109′	3·7	·00035
57′	18·8	·0042	64′	10·3	·0017	244′	10·2	·00041
100′	27·6	·0046	93′	17·4	·0020	—	—	—
135′	33·8	·0037	163′	25·3	·0018	—	—	—
316′	48·3	·0002	—	—	—	—	—	—

[1] This substance was not identified, but it has many of the properties of diethylene disulphide.

In each experiment the strength was the same, viz. 0·04 g. "sulphone," 5 cc. alcohol, 95 cc. $M/1000$ phosphate. Reaction p_H 7·0–7·4. In Exps. 2 and 3, k is calculated according to the equation $k = \frac{1}{t} \log_e \frac{a}{a-x}$. In these two experiments (where 1 % NaCl was present) it was impossible to obtain the end-point by the usual method. Even after three days, during which the flasks were kept at a temperature of 50–60°, the liberation of acid had only attained 50 % of the theoretical yield. The solutions, however, still retained their vesicant properties. The final reading was therefore obtained by calculating the yield of HCl from 0·04 g. of "sulphone." All three experiments can only approximate in accuracy to our experiments with DES, owing to the extreme slowness of the reaction. There is a tendency for k to fall off as the reaction proceeds.

DISCUSSION.

The acid theory of vesicant action.

If we compare the relative rate of acid production, at the same temperature reckoning DES as 100, the following approximate rates may be assigned:

A.	$\beta\beta'$-dichlorodiethyl sulphide	100	Vesicant
B.	$\alpha\alpha'$-dichlorodimethyl sulphide	500	Non-vesicant
C.	$\alpha\beta\beta'$-trichlorodiethyl sulphide	nil	,,
D.	$\alpha\beta\beta\beta'$-tetrachlorodiethyl sulphide	4	,,
E.	$\alpha\alpha\beta\beta\beta\beta'$-hexachlorodiethyl sulphide	1	,,
F.	$\beta\beta'$-dichlorodiethyl sulphone	1	Vesicant

Of this list only *A* and *F* are vesicant. All are soluble in both water and lipoid solvents. As *B* and *C* split off acid respectively faster and slower than DES *in vitro*, and are also non-vesicant, it is unlikely that their behaviour will be different in the cell. In this case, if acid liberation caused vesication, dichlorodimethyl sulphide should be a very powerful blisterer. It is non-vesicant. Turning to the other side of the picture, we have DES and its sulphone, of which the latter splits off acid about 100 times more slowly than DES. Both are vesicant, though the sulphone is less so than the sulphide[1]. It is evident that even if the production of acid has some adjuvant effect in vesication, we must look to some other property for the true causative factor. Further if we argue from the slowing effect of NaCl upon dichlorodiethyl sulphone *in vitro*, we are forced to conclude that in the cell the "sulphone" does not split off acid at all, and yet it is definitely vesicant. We think therefore that the "acid" theory must be dismissed.

The fact that a concentration of sodium chloride of 1 % practically inhibits the breakdown of dichlorodiethyl sulphone, as well as affording the strongest evidence against the acid theory of vesication, suggests that it is

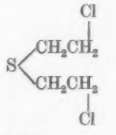
[1] Dr Dixon and Mr Gooding have told us in conversation that they have found the "sulphone" rather less vesicant than the "sulphide."

the chlorine-containing compound itself in the non-hydrolysed condition, which is responsible for the vesicant action. The experiments of Padtberg [1910] and others are of interest in this connection, because they have shown that (1) the skin is among the parts of the body which contain the greatest concentration of chloride, and (2) that the chloride concentration of the skin can be raised by drinking NaCl, the skin acting as Cl depot.

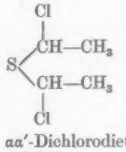
Introduction of Cl atoms into DES results in substances which do not readily liberate acid and which are non-vesicant. The acid theory has been dismissed. What other effect does the introduction of Cl atoms have which renders the compounds non-vesicant? Mann and Pope [1922] state that as the number of Cl atoms in the molecule of DES increases the S atom shows increasing reluctance to become quadrivalent and (in particular) that the facility with which the sulphoxide is formed diminishes. In short the chemical activity of the S atom is shut down. When the S atom in DES is replaced by O the resulting compound $\beta\beta'$-dichlorodiethyl ether is non-vesicant. Finally it may be recalled that allyl isothiocyanate (C_3H_5NCS) produces blisters when in contact with the skin. The only relation which this compound holds to DES is that it contains a sulphur atom.

The above evidence seems to indicate again that it is the unhydrolysed molecule which is the vesicant agent, and that the presence of a reactive "S" atom has an important bearing upon the vesicant power of these substances.

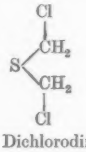
As an illustration of the complexity of the problem, it may be mentioned that $\alpha\alpha'$-dichlorodiethyl sulphide is devoid of vesicant properties. An inspection of the formula, however, will show that this compound bears a closer resemblance in structure to the non-vesicant dichlorodimethyl sulphide than to DES. The chlorine atoms are in the same position relative to the S atom in both these compounds.



DES



$\alpha\alpha'$-Dichlorodiethyl sulphide



Dichlorodimethyl sulphide

SOME CHEMICAL CONSIDERATIONS.

By analogy with esters of the dibasic acids, the hydrolysis of DES would be expected to proceed in two stages. There is actually chemical evidence of some difference between the chlorine atoms of the DES molecule; for instance Mann and Pope [1922] have shown that di-, tri-, tetra- and hexachloro-diethyl sulphide all contain the same group—S . CH_2 . CH_2Cl, *i.e.* upon chlorinating DES the additional Cl atoms all enter the same half of the molecule.

Russell [Report] has interpreted his results upon the basis of a two stage reaction. In a recent paper (published since the conclusion of this work)

Wilson, Fuller and Schur [1922] claim to have established the case for the two stage reaction; they further think that the second stage proceeds at least five times as fast as the first. Our results show that monomolecular "k" is not constant, and we think that they can only be interpreted upon the basis of a two stage reaction, viz.:

(1) $Cl \cdot X \cdot Cl + HOH \rightarrow OH \cdot X \cdot Cl + HCl.$

(2) $OH \cdot X \cdot Cl + HOH = OH \cdot X \cdot OH + HCl.$

We do not propose to attempt a derivation of kinetic formulae for the two stage reaction, a problem which can be more satisfactorily solved by the isolation and investigation of the intermediate compound. In our experiments chloride slowed the rate of acid liberation, within moderate limits OH-ion did not increase the rate, whereas increase in H-ion (produced by HCl) apparently slowed the rate. We have not investigated how far this effect is really due to increase in H-ion, or is an effect of increasing Cl-ion. Our method of experiment was not accurate at highly acid reactions. Since in our experiments the reaction and therefore the ionic environment has been kept constant, as regards the H- and OH-ions, we have to consider three essential stages, and the effect of Cl-ion upon them, viz.:

$$\underset{(A)}{Cl \cdot X \cdot Cl} \rightarrow \underset{(B)}{OH \cdot X \cdot Cl} \rightarrow \underset{(C)}{OH \cdot X \cdot OH}.$$

The driving force of the reaction may be considered to be derived from the difference in dissociation level between these three compounds. Since the reaction goes to completion, and under these conditions there is no recombination of "C" with HCl, compound "C" represents zero dissociation, compound "B" is intermediate between "A" and "C." Compound "B" in the main governs the rate of the reaction. The dissociation constant for this compound may be represented

$$k_1 = \frac{[X\langle^{OH}_{\ }][Cl']}{[X\langle^{OH}_{Cl}]}.$$

It will be seen that k_1 is unaffected by H$'$ and OH$'$. OH-ions are only important in so far as the stage $[X\langle^{OH}_{\ }] \rightarrow X\langle^{OH}_{OH}$ necessitates a sufficient supply of OH-ion. The dissociation of $X\langle^{OH}_{Cl}$ into the ions $X\langle^{OH}_{\ }$ and Cl$'$ will be sensitive to Cl$'$; increasing Cl$'$ will depress the dissociation of the intermediate compound, and will therefore act as a brake or chemical resistance upon the course of the reaction at this stage. This factor will also operate to a certain extent upon the dissociation of the first Cl atom from compound "A." There is left undiscussed the interesting problem as to why, out of the total number of DES molecules present, only a limited number are apparently undergoing change at a given instant. When the reaction is considered from the ionic

standpoint, the absence of depressant effect by $NaNO_3$ is explained. It is not however clear why Na_2SO_4 and $MgSO_4$ exerted such a depressant effect upon the later stages of the reaction, a fact which suggests that the sulphate affects only the second stage.

Since the chloride effect seems to be ionic in origin, it is pertinent to enquire whether the depressant effect of NaCl upon the course of the reaction as a whole with increasing concentrations is additive, or whether it follows the dissociation curve for NaCl solutions, obtained from conductivity data. Fig. 8 shows side by side the degree of depression in the characteristic constant produced by NaCl and the degree of dissociation of NaCl as judged from conductivity data (from Lewis). It will be seen that there is close correspondence, suggesting that the ionised part of the NaCl present is responsible for the depressant effect. We find this agreement difficult to reconcile with the theory of the complete dissociation of strong electrolytes (Bjerrum, quoted by [Warburg, 1922]).

It is also puzzling to find that Cl' does not interfere with the rate of reaction of dichlorodimethyl sulphide.

Chemical Constitution and Vesicant Action.

In connection with attempts to correlate chemical constitution and vesicant power, we feel that it is important to distinguish between (*a*) an initial disturbance to sensitive structures in the skin, and (*b*) the subsequent pathological changes. Many substances, unrelated chemically, such as cantharides, strong acids and radium will produce skin burns. Various initial stimuli therefore produce the final result of burn or blister. We feel that the attempt to relate chemical constitution and vesicant action can only be fruitful, if we consider groups of substances having the same initial effect. In a search for the mechanism by which DES irritates the skin, we are trying to find what common factor (chemical or physical) underlies the effect of the mustard gas group of vesicants; this is apparently not acid liberated in the cell. We do not think that it is a surface effect, because DES is not toxic to the protozoon *Colpidium*. Further the view that the "sulphide" is converted into the more stable "sulphone" by the action of skin oxidases [Flury and Wieland, 1921] is not supported by the greater activity of the "sulphide," or by the fact that the "sulphoxide" is non-vesicant. Rona and Petow [1920] claim that the "sulphide" and "sulphone" inhibit the action of urease, but they have also shown that the tetrachlorodiethyl sulphide, a non-vesicant, has a greater effect than the "sulphide." We are left with the possibilities that either (*a*) synthetic toxic products, or (*b*) breakdown toxic products are the effective mechanism initiating pathological change.

SUMMARY.

1. The theory that the intracellular liberation of acid is responsible for the vesicant action of dichlorodiethyl sulphide (DES), has been tested by studying the rates of acid liberation by the action of water upon DES and analogous compounds at constant reaction.

2. The rates of acid liberation in 5 % alcoholic solution, were (DES = 100), dichlorodimethyl sulphide 500, DES 100, tetrachlorodiethyl sulphide 4, hexachlorodiethyl sulphide 1, dichlorodiethyl sulphone 1, trichlorodiethyl sulphide 0.

3. In the case of DES, increasing concentrations of alcohol and of NaCl decrease the rate of acid liberation at constant temperature. The presence of NaCl does not slow the rate of acid liberation by dichlorodimethyl sulphide.

4. 1 % $NaNO_3$ does not influence the rate of acid liberation by DES. Sodium sulphate and magnesium sulphate inhibit the rate strongly in the later stages of the reaction.

5. Between 15° and 38°, the temperature coefficient of the reaction is 3·3 per 10° rise in temperature.

6. The velocity constant, k, of the reaction, calculated upon a monomolecular basis, shows generally a small but steady rise from 30–80 % hydrolysis. This rise is affected variously by different experimental conditions. The conclusion is drawn that the reaction is really a 2-stage reaction, as has been stated by other observers.

7. The acid theory is rendered untenable by the fact that vesicant action and rate of acid liberation do not run parallel in the series of compounds studied; any slight differences in lipoid solubility cannot be sufficient to counterbalance the large differences found in the rate of acid liberation.

We are indebted to Professor Sir Wm. Pope for the supply of the compounds used in this research. We are also indebted to Professor Hopkins for valuable discussion.

REFERENCES.

Boulin and Simon (1920). *Compt. Rend. Acad. Sci.* **170**, 845.
Flury and Wieland (1921). *Z. ges. exp. Med.* **13**, 367.
Hopkins, E. F. (1919). *J. Pharm. Exp. Ther.* **12**, 393.
Mann and Pope (1922). *J. Chem. Soc.* **121**, 594.
Marshall (1919). *J. Amer. Med. Ass.* **73**, 604.
—— (1920). *J. Pharm. Exp. Ther.* **16**, 259.
Lynch, Smith and Marshall (1918). *J. Pharm. Exp. Ther.* **12**, 291.
Moureu and Murat (1920). *Chim. et Ind.* **4**, 493.
Padtberg (1910). *Arch. exp. Path. Pharm.* **63**, 60.
Rona (1921). *Z. ges. exp. Med.* **13**, 16.
Rona and Petow (1920). *Biochem. Z.* **111**, 134.
Russell. Report to Amer. Chemical Warfare Section.
Wilson, Fuller and Schur (1922). *J. Amer. Chem. Soc.* **12**, 2867.
Warburg (1922). *Biochem. J.* **16**, 153.
Warthin and Weller (1919). The medical aspects of mustard gas poisoning.

XXXII. THE REVERSIBILITY OF THE ACTION OF UREASE OF SOY BEAN.

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(*Received March 19th, 1923.*)

Since Takeuchi in 1909 discovered that the soy bean contained urease in an easily available and very reactive form, many investigations have been made into the question of the mechanism of the hydrolysis of urea to ammonium carbonate by this enzyme, and several attempts have been made, on the basis of the now widely accepted view that the same enzyme which provokes hydrolysis must also produce synthesis, to show that urease is no exception to this general rule.

Specific reversibility of enzyme action, although shown to take place in the case of lipase by Kastle and Loevenhart [1900], Dietz [1907], Armstrong and Gosney [1914], and in the case of emulsin by Bayliss [1912], Bourquelot and Bridel [1913], is by no means so definitely established in the case of most other enzymes. Thus the synthetic action of invertase and the production of maltose from glucose by maltase, to mention only two cases in which specific reversibility has been claimed, are still matters of controversy. Any new direct evidence of specific synthetic power is therefore of considerable value.

Barendrecht [1920] in the course of a long investigation on the application of his radiation hypothesis to the case of urease, stated that this enzyme was reversible, adducing as his evidence that when soy bean meal is added to the first of two equal quantities of ammonium carbonate solution, less ammonia is given off from the former on treatment with potassium carbonate and aspiration through sulphuric acid. If more soy bean meal is added the shortage of ammonia increases, being roughly proportional to the amount of added meal. The actual difference was small, 2–4 parts per thousand of carbonate used. Urea was not isolated. Mattaar [1920] strongly criticised Barendrecht's conclusion, and using Fosse's xanthhydrol reaction for urea showed that urea was not produced under the conditions described by Barendrecht even to the one-fortieth part of the small extent that the latter had supposed. It was evident that if any direct synthesis of urea by this enzyme took place at all, the position of equilibrium was very near complete hydrolysis.

Fearon [1923] has detected and isolated cyanates in the reaction mixtures during the decomposition of an aqueous solution of urea by soy bean urease,

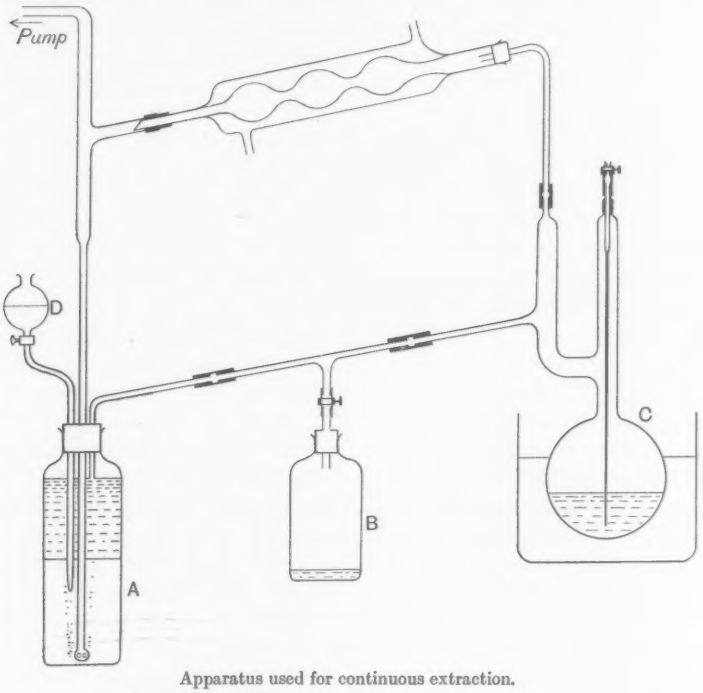
but he has been unable to find any evidence of the formation of urea from either ammonium carbonate or carbamate, and comes to the conclusion that the normal method of zymolysis of urea is by way of cyanic acid and ammonia, the former being rapidly hydrolysed by the solvent into more ammonia and carbon dioxide. Mack and Villars [1923, 1] have also isolated cyanates under similar conditions, but bring forward evidence [1923, 2] to show that when commercial urease is added to a very strong (10 *N*) solution of ammonium carbonate and carbamate at 55° (the optimum temperature for urease), small quantities of urea are produced and can be separated as the dixanthyl compound from the reaction mixture. They do not state whether any control experiments with heated inactivated urease were carried out, but find that a control without urease at the same temperature gives a very small precipitate with xanthhydrol.

There is no inherent chemical difficulty in the idea of the formation of urea from ammonium carbonate or carbamate. Lewis and Burrows [1912] showed that even at as low a temperature as 77°, on heating ammonium carbamate or urea in a sealed tube equilibrium was almost reached, after 95 days, at 1 % urea 99 % carbamate-carbonate. This same reaction at a higher temperature is described in a recent patent of the Badische Anilin und Soda Fabrik [1921] in which urea in 25 % yield is obtained by heating ammonium carbamate to 135°–140° under 15 atmospheres pressure.

But at ordinary temperatures and pressures this synthesis has not yet been observed, and the experiments about to be described indicate that the presence of urease is necessary if this synthesis is to take place at a measurable rate at room temperature.

The problem of showing that such a synthesis does take place in these circumstances is really that of separating urea in very minute quantity from a large excess of ammonium carbonate. The two substances are widely different in their chemical and physical properties, and separation may be achieved by taking advantage of the fact that whilst urea is soluble to the extent of 1·1 % in dry, and still more soluble in wet, butyl alcohol, ammonium carbonate is insoluble. Dakin's [1918, 1920] successful use of this partially miscible solvent in the continuous extraction of mono-amino acids from the products of protein hydrolysis led to my adopting it in an endeavour to effect this separation of urea. Since butyl alcohol boils at 118°, at which temperature small quantities of urea are rapidly decomposed, it was necessary to conduct the operation under reduced pressure, and a modification of the apparatus used by Dudley [1919] was employed. The temperature of the butyl alcohol was in this way kept at 60° during the course of the experiment, whilst a rapid stream of the solvent could be bubbled through the reaction mixture. This removed the urea from the reaction mixture almost as soon as it was formed, thus keeping its active concentration low and tending to force the reaction over towards further synthesis. The process of continuous removal from the sphere of reaction of substances synthesised by enzymes seems to

be a common biological phenomenon, and in this sense the method used in these experiments might be said to simulate what occurs in many living processes.



Apparatus used for continuous extraction.
A—reaction bottle.
B—bottle for excess butyl alcohol.
C—extraction flask containing butyl alcohol in bath at 60°.
D—dropping funnel containing urease.

Two preliminary experiments were made to find out whether butyl alcohol would extract urea quantitatively from its mixed solution with ammonium carbonate and carbamate:

(*a*) 1 g. of urea was dissolved in 100 cc. of a 10 % solution of dry ammonium carbonate, and extracted continuously for twelve hours with butyl alcohol. The residue in the extraction vessel was found to contain insufficient urea in 10 cc. to give a positive reaction with urease, which was easily given by 10 cc. of 0·05 % urea. Urea was shown to be present in the butyl alcohol in large quantities but was not estimated in this experiment. The extraction, then, is fairly complete.

(*b*) 0·001 g. of urea was dissolved in 100 cc. of a 20 % solution of ammonium carbonate, and extracted continuously for four periods of six hours

each, the separation and estimation of urea being conducted in the manner to be described later in this communication, using xanthhydrol.

		Wt urea extracted
First	six hour period	·0004 g.
Second	,, ,, ...	·00026 g.
Third	,, ,, ...	·00010 g.
Fourth	,, ,, ...	minute traces only

This gives a total quantity of about 75 % of the original urea extracted by this method from its very dilute solution in 18 hours.

By use of this apparatus, urea in small quantities has been isolated from the reaction mixture of urease + the equilibrium mixture of ammonium carbonate and carbamate which is obtained when strong ammonia solution is saturated with CO_2 gas at room temperatures and the resulting white crystalline precipitate is dissolved in distilled water. No urea whatever was obtained unless both active urease and this mixture were together present in the reaction bottle.

Experimental.

Ammonium carbonate. "Ammonium carbonate" was prepared by saturating portions of 250 cc. of 0·91 ammonia with dry CO_2 under a slight pressure. The precipitated salt was washed on a Buchner with a little cold distilled water, then with alcohol, and allowed to dry for two days in the air. On dissolving 20 g. of the dry solid in 100 cc. of water, some CO_2 was evolved and the resulting solution of p_H 8·2–8·4 was found to contain 84 % carbonate and 16 % carbamate, by Lewis and Burrows' [1912] method. During the extraction the p_H slowly increased, ending at 8·6–8·8, owing to loss of CO_2 from the solution.

Urease. Urease was prepared by two methods. In the earlier experiments it was obtained by extracting freshly ground soy bean meal with ten times its weight of 0·3 % KH_2PO_4 and allowing to stand for ten minutes. The mixture was then filtered through paper, allowed to stand for at least half-an-hour, and the activity of the urease was tested using a standard buffered solution of urea. It was then filtered into the dropping funnel shown on the left in the diagram, and covered by a layer of butyl alcohol to prevent any slight risk of bacterial contamination. Usually 20 cc. of the enzyme solution was used for a day's (8 hours) extraction, the liquid being added in 1 cc. portions at intervals. This procedure was adopted owing to the fact that butyl alcohol plus ammonium carbonate slowly destroy the enzyme.

Onodera [1915] showed that whilst in small concentrations the monohydric alcohols up to amyl alcohol increased the activity of soy bean urease (this increase being connected with the diminution in surface tension at the interface between the colloidal particles of the urease and the continuous phase), in higher concentrations the action was inhibitory. In a series of preliminary

trials the inhibitory effect of large concentrations of butyl alcohol was confirmed. Some idea of the relative activity of the enzyme before and after the experiment was obtained by noting the times taken by strictly comparable dilutions of the fresh enzyme and of the enzyme from the reaction mixture to change the p_H of a standard buffered solution of urea from 7·0 to 8·0. After a day's extraction the activity gauged in this way was diminished to one-fifth or less. Some of this effect was due to the ammonium carbonate, which on standing with urease for eight hours was found to diminish its activity, as previously observed by Barendrecht and others.

In the later experiments urease was prepared by Van Slyke and Cullen's method [1914] by two precipitations with ten volumes of acetone. The criticism that the urea found might have been present in some protected form in the cruder preparation could not arise in the case of this purified urease. 0·5 g. of the solid, dissolved in 20 cc. of distilled water, and filtered through glass wool was used for each day's (8 hours) extraction.

Methods of detection and analysis of urea. At the end of the period of continuous extraction, the stop-cock leading to the bottle *B* was opened and the butyl alcohol remaining in the extraction flask was distilled into this bottle until the flask was almost dry. The pressure was then released and the contents of the flask washed out with three washings of absolute alcohol into a small evaporating basin. The alcohol was evaporated on a water-bath and the dry residue washed three times with boiling chloroform. This removed most of the colouring matter which had come over in the butyl alcohol and left behind an almost colourless residue which was then extracted three times with 10 cc. of boiling acetone. The filtered acetone solution was evaporated to dryness, and the very small quantity of crystalline residue contained any urea which had been present in the extraction flask at the close of the extraction period. In later experiments it was found that the chloroform always extracted, together with the colouring matter, a trace of urea which could be detected by xanthhydrol precipitation but which was insufficient to weigh.

Two methods were used for the detection and estimation of the urea in the acetone extract.

1. The well-known method, by which the p_H of an indicator solution is changed to the alkaline side when urease of a known p_H and urea solution at the same p_H are added together and warmed to 37° for a short period, was extended to work with very small quantities of urea as follows:

A phosphate buffer mixture (*M*/15 KH_2PO_4 and *M*/15 $KNaHPO_4$) was made of $p_H = 7·0$, and 0·1 cc. of this was added to each of a series of tubes containing 5 cc. of dilute solutions of urea of different, known strengths with 0·5 cc. of 0·01 % phenol red.

Urease was prepared by diluting 5 cc. of the soya bean extract with an equal quantity of water, and adjusting the p_H to 7·0 with a few drops of *N*/50 NaOH. Of this enzyme solution 0·2 cc. was added to each of six tubes containing urea, the p_H immediately noted, and noted again at intervals.

Tube	Amount urea in mgm.	p_H at start	p_H after 15 mins. at 14°	p_H after raising to 37° for 15 mins.	p_H after 90 mins. at 37°
1	1·25	7·0	>8·4	>8·4	>8·4
2	0·25	7·0	7·8	7·9	7·9
3	0·10	7·0	7·35	7·4	7·4
4	0·05	7·0	7·1	7·1	7·15
5	0·025	7·0	7·0	7·1	7·1
6	0·012	7·0	7·0	7·0	7·0
7	1·25	Control with boiled enzyme no change of p_H			

This method is therefore capable of detecting 0·05 mgm. of urea in 5 cc. of liquid, 1 in 100,000, and in quantities somewhat greater than this the change of p_H gives a rough quantitative idea of the amount of urea present. Armstrong has shown how very specific the action of urease is, and this definite change of p_H after the addition of urease is convincing evidence of the presence of urea.

The advantage of this method was that it was possible to follow it by an estimation of the amount of ammonia produced from the synthesised urea by the enzyme, using a micro-Folin apparatus. Potassium carbonate was added to the reaction mixture and the ammonia liberated was aspirated into $N/100$ H_2SO_4 and titrated with $N/100$ CO_2-free soda in a CO_2-free atmosphere using methyl red as the indicator. The serious drawback was that half of the solution of urea had to be reserved for a blank determination with heated enzyme. It was always necessary to do this, as the blank gave from one-third to one-sixth of the total amount of ammonia produced by the active enzyme.

2. Xanthhydrol was used in the later experiments for the detection and estimation of urea using Nicloux and Welter's [1921] gravimetric modification of Fosse's well known method.

The acetone solution of urea obtained from the contents of the extraction flask in the usual way was evaporated to dryness and the minute crystalline residue dissolved in 2 cc. of water and filtered. The filter paper was washed with a little water, and to the 3 cc. of urea solution thus obtained were added 5 cc. of glacial acetic acid and 0·5 cc. of a 10 % solution of xanthhydrol in methyl alcohol. The liquid was shaken and allowed to stand at room temperature for ten minutes, when a crystalline precipitate could be observed uniformly throughout the tube. This began to separate, and after two to three hours the liquid was filtered off through a Neubauer micro-crucible and the dixanthyl urea washed and dried as described by Nicloux and Welter. The weight of urea is exactly one-seventh of the weight of the dixanthyl urea.

Results. These are tabulated below. About two-thirds of the experiments which have been done are given. Those omitted from considerations of space are duplicates completely confirmatory of the results shown.

The quantities of urea obtained are small, but quite recognisable and capable of estimation. In the later experiments as the technique improved the yields of urea became somewhat greater. Endeavours were made to obtain

Enzyme	Substrate	Time of extraction in hours	Method of testing for urea	Method of estimation	Amount urea found in mgm.
Soya bean extract	$(NH_4)_2CO_3$	6	Urease	Urease	>0·1
,,	Distilled water only	6	,,	,,	Nil
,,	$(NH_4)_2SO_4$[1]	6	,,	,,	,,
,,	$(NH_4)_2SO_4$ at $p_H = 8·6$	6	,,	,,	,,
,,	Distilled water	16	,,	,,	,,
,,	$(NH_4)_2CO_3$	18	,,	,,	0·3*
,,	,,	18	,,	Micro-Folin	0·2
None	,,	18	,,	Urease	Nil
,,	,,	20	Xanthhydrol	Xanthhydrol	,,
Soya bean extract boiled	,,	15	Urease	Urease	,,
Soya bean	,,	45	,,	Micro-Folin	>0·6
Van Slyke urease	,,	26	Xanthhydrol	Xanthhydrol	0·37
,,	,,	16	Urease	Micro-Folin	0·24
None	,,	20	Xanthhydrol	Xanthhydrol	Nil
Van Slyke urease[2]	,,	20	,,	,,	0·30
,,	,,	12	,,	,,	0·35

[1] Of same concentration in NH_4 ions as the ammonium carbonate used in the same series of experiments.

[2] A particularly active preparation.

* Reaction mixture in bottle kept at 37° during last eight hours of this experiment.

larger quantities of urea by conducting the operation in strong cane sugar solutions, and thus forcing the simple reaction

$$CON_2H_4 + 2H_2O \rightleftarrows CO_3 . (NH_4)_2$$

over towards the side of synthesis by diminishing the amount of available water, but the results were not encouraging:

Enzyme	Substrate	Time of extraction in hours	Method of testing and estimation	Amount of urea found in mgm.
Soya bean extract	$(NH_4)_2CO_3$	8	Urease	0·1
,,	$(NH_4)_2CO_3$ in 25 % cane sugar	14	,,	0·1
,,	$(NH_4)_2CO_3$ in 50 % cane sugar	20	,,	Traces only

No further attempts have been made, up to the present, to increase the yield.

The actual change in colour of the indicator, on hydrolysis of half of the urea solution (the other half being kept as control) by means of the same enzyme that had synthesised it, was most striking, particularly with the larger quantities of urea. In the "synthesised" urea solution the rate of colour change was the same as in a solution of urea of the same approximate strength made up for comparison.

In two experiments not given above the synthesised urea was crystallised from a very small quantity of acetone (by evaporation) and compared microscopically with a similar quantity of ordinary urea similarly treated. The resemblance of crystalline form was evident. A small portion of the synthesised urea placed on the outside of a test-tube containing boiling butyl alcohol (118°) did not melt, but when put onto the outside of a test tube in which sulphuric acid was being heated melted below 126°. (Urea melts at 132°.) There was insufficient for a M.P. determination in the ordinary way. The urea

still contained a little pigment. On heating in a small dry tube, ammonia was given off, but as this reaction meant the disappearance of the yield of 18 hours' extraction it was not repeated.

Discussion.

It has been established by these experiments that urea may be extracted from a solution containing *active* urease and ammonium carbonate-carbamate mixture, but not under conditions in which the enzyme is destroyed, or in which either the ammonium carbonate or the active enzyme is absent.

Control experiments as indicated above have shown that the urea does not come from

(*a*) ammonium carbonate + carbamate alone;
(*b*) the enzyme solution alone;
(*c*) the enzyme in presence of another ammonium salt;
(*d*) the inactivated enzyme in presence of ammonium carbonate and carbamate.

The control experiments also negative the possibility that the urea found is derived from the oxidation of the butyl alcohol in presence of ammonia to cyanic acid and the transformation of this substance into urea [cf. Fosse and Laude, 1921]. A solution of ammonium sulphate made alkaline with ammonia in presence of active urease was found to give no trace whatever of urea under the same conditions of experiment in which ammonium carbonate gave easily recognisable quantities.

A fair summary of the situation with regard to the various reactions which may theoretically take place during the hydrolysis and synthesis of urea is given by Mack and Villars [1923, 1], and the experiments described above fall most easily into line with the conclusion of these authors that the reaction urea $\rightleftarrows$ ammonium carbamate is the one catalysed by urease and not the reaction urea $\rightleftarrows$ ammonium cyanate, or urea $\rightleftarrows$ cyanic acid + ammonia. In the latter case it would be necessary also for the urease to catalyse the reaction cyanic acid + ammonia $\rightleftarrows$ ammonium carbonate which, as has been known for some time [Walker and Hambley, 1895], is a very slow reaction in alkaline solution even at 69°, and at equilibrium only gives about 4 % of ammonium carbonate. The great rapidity and the completeness with which urea is hydrolysed by urease would therefore seem, in themselves, to put this explanation out of court.

Further experiments are being carried out in an endeavour to throw more light on the mechanism of the action of urease.

Summary.

Urea is produced in very small quantities during the action of urease on a mixture of ammonium carbonate and carbamate in strong solution in water, at room temperatures, under conditions which preclude the formation of urea by any other means than enzymic synthesis. The action of urease is, therefore, reversible.

REFERENCES.

Armstrong and Gosney (1914). *Proc. Roy. Soc.* B. **88**, 176.
Badische Anilin und Soda Fabrik (1921). *Chem. Zentr.* **11**, 647.
Barendrecht (1920). *Rec. trav. chim.* **39**, 59.
Bayliss (1912). *J. Physiol.* **43**, 455.
Bourquelot and Bridel (1913). *Ann. chim. phys.* **28**, 145.
Dakin (1918). *Biochem. J.* **12**, 290.
—— (1920). *J. Biol. Chem.* **44**, 512.
Dietz (1907). *Z. physiol. Chem.* **52**, 279.
Dudley (1919). *J. Pharmacol.* **14**, 295.
Fearon (1923). *Biochem. J.* **17**, 84.
Fosse and Laude (1921). *Compt. Rend. Acad. Sci.* **172**, 684.
Kastle and Loevenhart (1900). *J. Amer. Chem. Soc.* **24**, 491.
Lewis and Burrows (1912). *J. Amer. Chem. Soc.* **34**, 1515.
Mack and Villars (1923, 1). *J. Amer. Chem. Soc.* **45**, 505.
—— —— (1923, 2). *J. Amer. Chem. Soc.* **45**, 501.
Mattaar (1920). *Rec. trav. chim.* **39**, 495.
Nicloux and Welter (1921). *Compt. Rend. Acad. Sci.* **173**, 1490.
Onodera (1915). *Biochem. J.* **9**, 562.
Van Slyke and Cullen (1914). *J. Biol. Chem.* **19**, 211.
Walker and Hambley (1895). *J. Chem. Soc.* **67**, 750.

XXXIII. THE POSSIBLE SIGNIFICANCE OF HEXOSEPHOSPHORIC ESTERS IN OSSIFICATION.

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Among the esters of phosphoric acid possessing biochemical interest three are known in which the phosphoric acid group is believed to be combined with a hexose molecule.

Hexosediphosphoric acid was discovered by Harden and Young [1905] and by Ivanov [1905] among the products of the fermentation of glucose, fructose or mannose by yeast juice in presence of phosphates, and was shown by Young [1909, 1911] to possess the constitution $C_6H_{10}O_4(PO_4H_2)_2$ and to yield fructose on hydrolysis. One of the two phosphoric acid molecules is very easily removed by boiling the ester with weak acids and in this way Neuberg [1918] obtained a hexosemonophosphoric acid $C_6H_{11}O_5(PO_4H_2)$ which like the diphosphoric ester is very slightly dextrorotatory and yields a laevorotatory sugar on further hydrolysis.

The presence of a hexosemonophosphoric ester was observed by Harden and Robison [1914] among the products of fermentation of sugar by yeast juice, and the isolation and investigation of this ester has been described by Robison [1922] in a recent number of this Journal. It is entirely different from the compound prepared by Neuberg, being strongly dextrorotatory and yielding a dextrorotatory sugar on hydrolysis. The phosphoric acid group in this ester is moreover much more slowly removed by boiling with acids than either of the groups in hexosediphosphoric acid.

The barium and calcium salts of the diphosphoric ester are sparingly soluble in cold and still less soluble in hot water. Those of the monophosphoric esters are on the contrary very easily soluble in water.

In animal physiology hexosediphosphoric acid has acquired great interest and significance through the researches of Embden and his co-workers who have brought forward convincing evidence [Embden and Lacquer, 1921] that this or some closely related phosphoric ester is present in striped muscle and is identical with the "lactacidogen."

During my investigation of the hexosemonophosphoric acid isolated from the products of fermentation, the hydrolysis of the ester by enzymes was studied. In some experiments in which the readily soluble calcium and barium

salts were used as substrate, the progress of the hydrolysis was shown by the formation of a precipitate of sparingly soluble calcium or barium phosphates,

$$C_6H_{11}O_5PO_4Ca + H_2O \rightarrow C_6H_{12}O_6 + CaHPO_4.$$

The formation of this precipitate suggested to me the query whether some such reaction might conceivably be concerned in the deposition of calcium phosphate during the formation of bone in the animal body. In the first instance I sought for the presence of an enzyme capable of effecting such hydrolysis in the bones of growing animals.

Experiment 1. *Aqueous extract of bone of rabbit.* The femur taken from a young rabbit immediately after death was pounded with water saturated with toluene and after two hours the extract was filtered. 0·1 cc. of this extract, equivalent to 0·02 g. fresh bone was incubated at 37° with 2 cc. of a 1 % solution of barium hexosemonophosphate + 0·05 cc. toluene. Controls containing (1) 0·1 cc. extract in 2 cc. water, (2) 2 cc. barium hexosemonophosphate solution without extract, were incubated at the same time. Within 18 hours a precipitate of barium phosphate had formed in the solution containing the phosphoric ester + bone extract but not in either of the controls. After 60 hours at 37° the free phosphate in all three tubes was estimated, the results indicating that 35 % of the total phosphoric acid had been hydrolysed by the bone extract.

The free phosphoric acid was estimated by precipitation with ammonium molybdate as in Neumann's method, omitting the previous ashing with nitric and sulphuric acid. The exact method employed was as follows:—A solution containing 5 cc. concentrated sulphuric acid, 25 cc. of a 50 % solution of ammonium nitrate, and 60 cc. water is heated to 75°–80°. The phosphate solution to be estimated is then added, followed immediately by 20 cc. of a 10 % solution of ammonium molybdate. If the volume of the phosphate solution is much greater than 10 cc. the amount of water is correspondingly reduced. The temperature of the solution before the addition of the ammonium molybdate should be 70°–75°. The flask is vigorously shaken and allowed to stand for $\frac{1}{4}$–$\frac{1}{2}$ hr. The yellow precipitate is then filtered off in the special funnel described by Plimmer and Bayliss [1906], washed with ice water and estimated in the usual way with standard NaOH solution, 0·1*N* solution being employed when the amount of free phosphoric acid corresponds with less than 2 mg. P. Carbon dioxide must be expelled by adding a slight excess of acid and boiling for a few minutes before the final titration.

With this method very varying quantities of phosphorus can be estimated with an accuracy of about 0·01 mg. (using 0·1*N* solution) or indeed, as Iversen [1920, 1, 2, 3] has shown, a still greater degree of accuracy may be obtained if special precautions are taken.

It cannot, of course, be taken for granted that no organic ester of phosphoric acid will be hydrolysed under the above conditions, but actual tests showed that with solutions of hexosemonophosphoric acid, glycerophosphoric

acid and even hexosediphosphoric acid, the amount of hydrolysis is negligible provided the temperature is not allowed to rise or the time prolonged unduly.

The total phosphorus was estimated in all cases by Neumann's method.

Experiment 2. *Bones of a young rat* (age 16 days, weight 28 g.). The epiphyseal cartilage was removed from both ends of each bone. The cartilage and shaft from one femur, tibia and humerus were each separately incubated 22 hours at 37° with 1 cc. of a solution of potassium hexosemonophosphate (sol. A) + 0·05 cc. chloroform. The cartilage and shaft of the second femur and tibia and the shaft of the humerus were incubated with 1 cc. water + 0·05 cc. chloroform. The cartilage of the second humerus was heated to 100° for a few minutes and then incubated with solution A. The weight of bone or cartilage in each tube was not accurately determined but was about 0·05 g.

Experiment 3. *Bones of a rachitic rat.* These were treated as described above—cartilage and shaft being separately tested.

The results of experiments 2 and 3 are shown in Table I.

Table I.

Experiment	Total P mg. P in 1 cc. sol. A	Free PO_4 after 22 hours at 37° (mg. P)				Percentage of combined P hydrolysed by tissue
		1 cc. sol. A	Tissue + water	Tissue + sol. A	Difference	
2. Normal rat:						
Femur shaft	3·53	0·10	0·00	1·76	1·66	48
„ cartilage	„	„	0·00	1·16	1·06	31
Tibia shaft	„	„	0·00	1·86	1·76	51
„ cartilage	„	„	0·00	1·35	1·25	36
Humerus shaft	„	„	0·00	1·27	1·17	34
„ cartilage	„	„	—	1·33	1·23	36
Humerus cartilage previously heated to 100°	„	„	—	0·09	−0·01	0
3. Rachitic rat:						
Humerus shaft	3·72	0·10	0·03	2·60	2·47	68
„ cartilage	„	„	0·07	2·95	2·78	77

These results clearly demonstrate the presence of an enzyme which can effect the hydrolysis of hexosemonophosphoric acid in the bones and ossifying cartilage of young rats and certainly not less in those of rachitic than those of normal animals. The next step was to discover whether the enzyme is present in other tissues also. For this purpose a young rat from the same litter as those used in the previous experiments was taken.

Experiment 4. *Tissues of young rat* (age 30 days, weight 48 g.). Weighed quantities (0·05 g. ± 5 %) of the various tissues were removed with all precautions against bacterial contamination and were incubated with 1 cc. of potassium hexosemonophosphate solution + 0·05 cc. chloroform (series A) or with 1 cc. of water + 0·05 cc. chloroform (series B). After 46 hours at 37° the contents of each tube were diluted with 5 cc. distilled water and filtered through a small Buchner funnel, the tube and funnel being rinsed out three times with 5 cc. water. Free phosphate was estimated in the filtrate and washings. The results are given in Table II. In the case of the spleen and

trachea the weight of tissue available did not permit of 0·05 g. being taken and the amount of phosphoric acid formed by hydrolysis has therefore been multiplied by the appropriate factor in order that the figures (enclosed in brackets) may be compared with those for the other tissues.

Table II.

Tissue	Weight g.	Total P mg. in 1 cc. sol. A	Free PO_4 after 46 hours at 37° (mg. P) 1 cc. sol. A	Tissue + water	Tissue + sol. A	Difference	Percentage of combined P hydrolysed by tissue
Femur cartilage (epiphysis)	·05	3·72	0·16	0·10	3·47	3·21	90
Rib cartilage	·05	,,	,,	0·08	0·30	0·06	2
Trachea	·02	,,	,,	0·04	0·30	0·10 (0·25)	3 (7)
Liver	·05	,,	,,	0·06	0·62	0·40	11
Spleen	·04	,,	,,	0·06	0·45	0·23 (0·29)	7 (8)
Thymus	·05	,,	,,	0·28	0·50	0·06	2
Heart muscle	·05	,,	,,	0·07	0·55	0·32	9
Pancreas	·05	,,	,,	0·21	0·48	0·11	3
Kidney	·05	,,	,,	0·15	2·59	2·28	64

The figures in the last column give the percentage of combined phosphoric acid set free by the tissue enzyme and point to an almost unique position for ossifying as distinct from non-ossifying cartilage in respect of this enzyme. Of the other tissues, though slight hydrolysis was produced in all cases, only the kidney approached the femur epiphysis in enzymic power. The trachea and rib cartilage are particularly interesting as examples of non-ossifying cartilage, both showing a very low hydrolytic power. In this experiment the ribs themselves were not examined nor the costachondral junctions, the cartilage being specially taken at some distance from the latter, but in a later experiment the costachondral junctions were found to contain the enzyme in a high degree and to be entirely comparable with cartilage of other bones in this respect.

In all the above experiments salts of hexosemonophosphoric acid were employed as substrate. In the next the action of various tissues on salts of hexosemonophosphoric, hexosediphosphoric and of glycerophosphoric acid was compared.

Experiment 5. *Tissues of rabbit* (age under 4 weeks). The tissues were macerated with twice their weight of water containing 2 % of chloroform until a fairly uniform mixture was obtained. 0·3 cc. of this maceration mixture, equivalent to 0·1 g. of fresh tissue was placed in each of the following solutions:

Sol. 0. 2 cc. water + 0·1 cc. chloroform
Sol. 1. ,, potassium hexosemonophosphate sol. (combined P = 5·47 mg.) + 0·1 cc. chloroform
Sol. 2. ,, ,, hexosediphosphate sol. (combined P = 4·00 mg.) + 0·1 cc. ,,
Sol. 3. ,, ,, glycerophosphate sol. (combined P = 6·58 mg.) + 0·1 cc. ,,

All solutions were incubated for 40 hours at 37° and were then filtered and the free phosphate estimated as described above. 2 cc. of sols. 1, 2 and 3 were also incubated with 0·1 cc. chloroform but without the addition of any tissue, as controls. The traces of free phosphate present in these solutions were not increased by the incubation, indicating that no appreciable hydrolysis takes

place in the absence of tissue under these conditions. To avoid multiplying the number of columns these small amounts have been subtracted from the corresponding totals and are not shown in the table (Table III). The amount of free phosphate arising from autolysis of the tissues is given in the first column, and this has also been subtracted from the totals in order to arrive at the amount of phosphoric ester hydrolysed by the tissue, as shown in the remaining columns.

Table III.

	Free phosphate after 40 hours' incubation at 37° (mg. P)				Percentage of phosphoric ester hydrolysed by tissue		
Tissue	Tissue + sol. 0 (autolysis)	Tissue + sol. 1 less P in controls	Tissue + sol. 2 less P in controls	Tissue + sol. 3 less P in controls	Sol. 1 potassium hexosemono-phosphate	Sol. 2 potassium hexosedi-phosphate	Sol. 3 potassium glycero-phosphate
Femur cartilage (epiphysis)	0·13	5·19	3·56	3·64	95	89	56
Rib cartilage	0·01	0·45	2·14	0·39	8	54	6
Trachea cartilage	0·11	—	1·31	—	—	33	—
Blood	0·03	0·02	0·01	0·53	0	0	8
Liver	0·15	1·19	2·62	1·54	22	66	24
Spleen	0·22	1·18	1·98	0·89	22	50	14
Pancreas	0·20	0·31	0·92	0·37	6	23	6
Muscle	0·17	0·08	1·98	0·15	1	50	2
Kidney	0·16	2·22	3·17	1·33	41	79	20

The figures for sol. 1 (potassium hexosemonophosphate) follow in general the same order as those in Table II, and show the same striking superiority of the epiphyseal cartilage over other tissues with respect to this enzyme. The hydrolysis of the ester by the femur cartilage is almost complete while the kidney, which is again second, only hydrolysed 41 %. Liver and spleen were relatively rather more active than in the previous experiment while with muscle and blood practically no hydrolysis was shown. A somewhat similar order is found in the figures for sol. 3 (potassium glycerophosphate) though the degree of hydrolysis is usually less than with hexosemonophosphate[1]. Whether the somewhat pronounced variations between the two sets of figures, *e.g.* in those for liver and blood, indicate that a different enzyme is involved, can only be decided by further experiments. The action of tissue enzymes on glycerophosphates has been investigated by Grosser and Husler [1912] who found that kidney and intestine effected complete hydrolysis in 24 hrs. at 37°, liver only 16 %, while spleen, blood and muscle were inactive. Plimmer [1913] obtained similar results with kidney and intestine, but found extracts of liver and pancreas to be inactive. In these as in my own experiments the glycerophosphate employed was the commercial preparation and probably contained both α and β varieties. The results obtained by these investigators and my own (including experiments not recorded in this paper) point to a variable but usually very low hydrolytic power for liver and liver extracts with respect to both glycerophosphoric acid and hexosemonophosphoric acid. The figures for sol. 2 (potassium hexosediphosphate) in Table III

[1] The difference in the rate of hydrolysis of potassium hexosemonophosphate and potassium glycerophosphate has not been confirmed in later experiments and may have been due to slight differences in the p_H of the two solutions. (Note added April 13th.)

differ considerably from those for the other esters and appear at first sight to show no striking variations among themselves. On closer examination however, one interesting fact emerges, that nearly all the tissues examined possess an enzyme capable of hydrolysing up to 50 % of the combined phosphorus—*i.e.* one of the two phosphoric acid groups. Thus muscle and rib cartilage, which are almost without action on hexosemonophosphoric acid and glycerophosphoric acid both hydrolyse approximately 50 % of the phosphorus in the diphosphoric ester. Only blood is entirely inactive. The hydrolysis of the second phosphoric acid group appears then to follow approximately the same order as that for hexosemonophosphoric acid. Obviously two different enzymes are here involved. The very different hydrolytic power of muscle with regard to the two phosphoric acid groups is significant in view of the close relationship between hexosediphosphoric acid and lactacidogen.

Tomita [1922, 1 and 2] has also investigated the action of animal tissues on the synthetic sucrose monophosphate, and on Neuberg's hexosemonophosphate and has found them to possess hydrolytic power diminishing in the following order: kidney, liver, spleen, pancreas, muscle.

Discussion of Results.

It has thus been shown that the ossifying cartilage of young rats and rabbits contains a very active enzyme which rapidly hydrolyses hexosemonophosphoric ester and also glycerophosphoric ester. The kidney also contains this enzyme but its hydrolytic power is considerably less, for equal weights of tissue, than that of ossifying cartilage. Other tissues examined, including non-ossifying cartilage from ribs and trachea, contain the enzyme in very much smaller degree or not at all.

The possible significance of these facts in processes of ossification may now be considered. The cause of the deposition of calcium phosphate in the growing bone and its non-deposition elsewhere has not been explained. It has been calculated [Adler, 1908] that an aqueous solution containing the same amounts of inorganic salts as are found in serum would deposit a mixture of calcium phosphate and carbonate. The increased solubility of these salts in presence of serum proteins has been given [Pauli and Samec, 1909] as an explanation of the non-formation of such a precipitate in blood while specific adsorption of ions by the cartilage has been suggested [Pfaundler, 1904] to explain the deposition of calcium salts in bone. It has been shown by many investigators that blood contains in addition to lipoid phosphorus and inorganic phosphate a certain amount of combined phosphoric acid which is readily hydrolysed by boiling with acids. Thus Greenwald [1916] found a small amount of such combined phosphoric acid (less than 1 mg. P per 100 cc.) in serum. Bloor [1918] found similar amounts of organic phosphate (10 % of the total phosphates) in plasma of normal men and women but much greater amounts (60–80 % of the total phosphates) in the corpuscles. Jones and Nye [1921] have obtained similar figures for corpuscles from the blood of children of both sexes but

think that the unknown phosphoric acid in plasma is negligible in amount. In a later paper McKellips, De Young, and Bloor [1921] give analyses of blood of very young infants and find that the amount of this unknown phosphoric acid in the plasma is much higher (average 2·2 mg. P per 100 cc.) than for adults.

From a consideration of the work of these investigators it appears very probable that some ester of phosphoric acid which is soluble in acids but insoluble in ether and which is not precipitable by magnesia mixture or by ammonium molybdate is present in small amounts in plasma and in relatively large amounts in the corpuscles. If this ester is hydrolysable by the enzymes of ossifying cartilage the result would be an increase of HPO_4'' ions in the cartilage. If a condition of equilibrium exists between solid calcium phosphate on the one hand and a solution containing un-ionised calcium phosphate, and calcium and phosphate ions on the other, any increase in the concentration of either of these ions will result in further deposition of the solid calcium phosphate.

I would therefore suggest that the hydrolysis of some phosphoric ester, the calcium salts of which are readily soluble in water, by the enzyme in the ossifying cartilage, with production of free phosphoric acid may possibly be a factor in the process of bone formation. Whether this ester is a hexose-monophosphoric acid can only be decided by its isolation and this I am attempting to carry out. In conjunction with Mr H. D. Kay, experiments have already been performed which indicate with considerable probability that the "acid soluble" organic phosphate of blood is hydrolysed by the ossifying cartilage of young animals. The problem is also being attacked in another way with promising results. A preliminary experiment may be briefly described.

Bones from a severely rachitic rat were split lengthwise and one-half of each immersed in a solution of calcium hexosemonophosphate—the other half being immersed in normal saline. Both were incubated at 37° for two days in presence of chloroform and were then removed, washed with distilled water and fixed in formalin. Sections of these bones were made and stained with silver nitrate. The half which had been incubated with saline showed the customary greatly enlarged zone of provisional calcification practically devoid of phosphate while the half bone which had been incubated with the calcium hexosemonophosphate solution showed an intense black stain in the matrix of the cartilage cells when treated with silver nitrate. This may conceivably have been due merely to adsorption of the ester, but it was noted that the deposit occurred only in and throughout the depth of the zone of provisional calcification and not in the proliferating cartilage as might have been expected if adsorption were the sole cause. Further experiments must decide this point. For the histological investigations, as well as for the supply of the rachitic bones and for advice on this aspect of the work I am very greatly indebted to the kindness of Dr H. Goldblatt to whom I would here express my thanks.

SUMMARY.

An enzyme is present in the ossifying cartilage of young rats and rabbits, which rapidly hydrolyses hexosemonophosphoric acid, yielding free phosphoric acid.

In respect of this enzyme the kidney is considerably less active (about 50 %) than an equal weight of epiphyseal cartilage. Other tissues contain the enzyme in a very much lower degree, muscle and blood being almost inactive.

Non-ossifying cartilage shows less than one-tenth of the hydrolytic power of ossifying cartilage.

The same tissues in approximately the same order also hydrolyse glycerophosphoric acid.

One of the two phosphoric acid groups of hexosediphosphoric acid is very readily hydrolysed by almost all tissues (including muscle and non-ossifying cartilage) except blood.

The possible significance of this enzyme in the process of ossification in the animal body is discussed and certain preliminary experiments in various directions are briefly described, by the further prosecution of which it is hoped to obtain more information on this subject.

REFERENCES.

Adler (1908). *J. Amer. Med. Ass.* **2**, 752.
Bloor (1918). *J. Biol. Chem.* **36**, 49.
Embden and Lacquer (1921). *Z. physiol. Chem.* **113**, 1.
Greenwald (1916). *J. Biol. Chem.* **25**, 431.
Grosser and Husler (1912). *Biochem. Z.* **39**, 1.
Harden and Robison (1914). *Proc. Chem. Soc.* **30**, 16.
—— and Young (1905). *Proc. Chem. Soc.* **21**, 189.
Ivanov (1905). *S. Travaux de la Soc. des Naturalistes de St Petersbourg*, 34.
Iversen (1920, 1). *Biochem. Z.* **104**, 15.
—— (1920, 2). *Biochem. Z.* **104**, 22.
—— (1920, 3). *Biochem. Z.* **109**, 211.
Jones and Nye (1921). *J. Biol. Chem.* **47**, 322.
McKellips, De Young and Bloor (1921). *J. Biol. Chem.* **47**, 53.
Neuberg (1918). *Biochem. Z.* **88**, 432.
Pauli and Samec (1909). *Biochem. Z.* **17**, 235.
Pfaundler (1904). *Jahrb. Kinderheilk*, **60**, 123.
Plimmer (1913). *Biochem. J.* **7**, 43.
Plimmer and Bayliss (1906). *J. Physiol.* **33**, 439.
Robison (1922). *Biochem. J.* **16**, 809.
Tomita (1922, 1). *Biochem. Z.* **131**, 161.
—— (1922, 2). *Biochem. Z.* **131**, 170.
Young (1909). *Proc. Roy. Soc.* B. **81**, 528.
—— (1911). *Biochem. Z.* **32**, 178.

XXXIV. A STUDY OF RATS ON A NORMAL DIET IRRADIATED DAILY BY THE MERCURY VAPOUR QUARTZ LAMP OR KEPT IN DARKNESS.

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Historical.

In the course of a study on the absence of rickets during confinement on a good diet, Mellanby [1921] kept four puppies in small kennels in the dark for a period of thirteen weeks. Their diets consisted of:

(*a*) bread, milk and meat;

(*b*) bread, milk, meat and bone;

(*c*) bread, milk, meat and bone that had been autoclaved at 120° for 15 minutes.

The puppies were ten weeks old at the beginning of the experiment and were killed at the age of 26 weeks. Histologically, no sign of rickets was found. Chemical analysis of the shafts of the femurs gave the following percentages of CaO in the dry bone:

Puppy No.	25	... ...	42·92
,,	26	... ...	38·13
,,	27	... ...	37·46
,,	28	... ...	30·00

These values Mellanby considers to be high. It is evident from the foregoing facts that the calcium content of the bones was unaffected by 13 weeks of confinement in the dark.

Raczynsky [1912] investigated the effect of sunlight and darkness on puppies during the period of lactation. He took two puppies from the same litter, born in May, kept one in total darkness from birth and the other in sunlight, both suckled by the same mother. After six weeks they were killed

and the bodies chemically analysed. The result is given in the following table:

	Per 100 g. of body weight	
	Puppy in sunlight	Puppy in darkness
	g.	g.
CaO	1·58	0·98
P_2O_5	1·19	0·86
MgO	0·05	0·04
Cl	0·16	0·35
Fe	0·02	0·02

From the fact that the calcium and phosphorus content of the puppy kept in the dark was lower than that of the one kept in sunlight, he surmised that the former had rickets. The diet of the mother is not known, therefore it cannot safely be assumed that the puppies received an optimal amount of calcium, phosphorus and anti-rachitic organic factor. Comparing the results with those about to be recorded, one ventures to suggest that the mother's diet probably was not complete.

Experimental.

Radiation. Since sunlight was not available, the radiations from a mercury vapour quartz lamp were used as a substitute. The lamp employed is a Pistolette, manufactured by the Hewittic Electric Co. The shortest wave length of its spectrum is 230 $\mu\mu$ and a daily twenty minute exposure to the rays from this lamp has been found to prevent or cure rickets in rats on diet No. 3143 of McCollum and his collaborators [1921], and on other rickets-producing diets.

Two litters of rats were divided into two groups with an equal number of the same sex in each. One group was kept in total darkness from the time the rats were weaned, while the other was kept in a room with a large double window consisting of plate glass about half-an-inch thick, through which daylight had to filter to enter the room. But the latter group was exposed for 20 minutes daily at a distance of 60 cm. to the radiations from the mercury vapour quartz lamp.

Diets. In the case of litter XXI, both groups received the following diet:

Diet N 1	Inactivated commercial "casein" ...	20·0 g.
	Starch (wheat)	50·0
	Cotton-seed oil (hardened)	10·0
	Cod-liver oil	5·0
	Salt mixture (McCollum No. 185) ...	5·0
	Calcium carbonate	3·1
	Marmite	5·0
	Lemon juice	5·0 cc.
	Distilled water	46·9 cc.

In the above diet the percentage of calcium in the paste was 1·07, of phosphorus 0·53 and of cod-liver oil 3·66, all optimal amounts for these substances.

The two groups of litter XXII were subdivided so that half the rats in each group (darkness and light) received the same diet as litter XXI and the other half a similar diet in which butter was substituted for both the cod-liver and cotton-seed oils. This diet consisted of:

Diet N 2	Inactivated commercial "casein" ...	20·0 g.
	Starch (wheat)	50·0
	Fresh butter	15·0
	Salt mixture (No. 185)	5·0
	Calcium carbonate	3·1
	Marmite	5·0
	Lemon juice	5·0 cc.
	Distilled water	46·9 cc.

The rats of litter XXI were 23 days and those of litter XXII 28 days old at the beginning of the experiment. At the end of eight weeks both groups were killed. That experimental period was chosen as it was the one used most frequently in other investigations and because the rats were then about twelve weeks old and therefore nearly full-grown.

Table I.

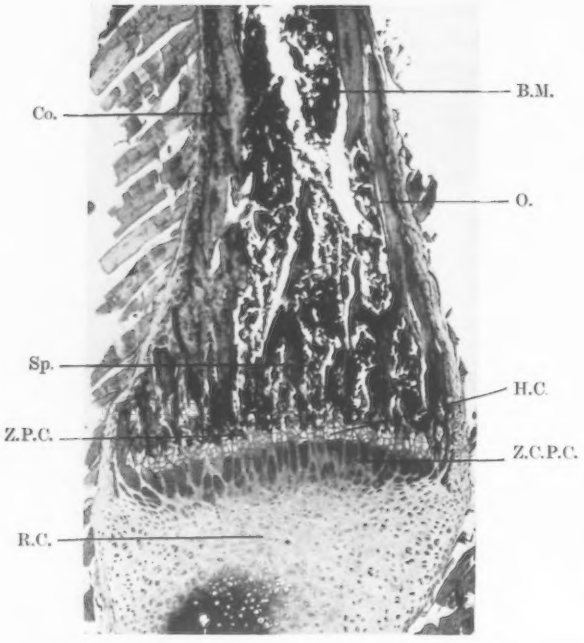
No. of rat	Sex	No. of litter	Diet	Age (in days) Initial	Final	Days on diet	Average amount of food eaten daily	Weight grams Initial	Final	Gain grams	In bones % H_2O	% Ca wet	% Ca dry
G. 409	♀	XXI	*N* 1	23	79	56	12·6	28	101	73	40·95	12·51	21·21
G. 410	♂	XXI	*N* 1	23	79	56	17·2	34	172	138	40·10	12·67	21·15
G. 411	♂	XXI	*N* 1	23	79	56	16·0	38	161	123	40·88	12·51	21·13
										Averages	40·64	12·56	21·16
G. 412	♀	XXI	*N* 1	23	79	56	12·2	33	113	80	38·25	12·80	20·73
G. 413	♂	XXI	*N* 1	23	79	56	15·0	33	139	106	38·12	12·72	20·55
G. 414	♂	XXI	*N* 1	23	79	56	17·5	38	160	122	42·28	12·22	21·15
										Averages	39·55	12·58	20·81
G. 424	♀	XXII	*N* 1	28	84	56	14·5	31	123	92	41·46	13·05	22·28
G. 425	♂	XXII	*N* 1	28	84	56	21·1	36	198	162	40·00	13·61	22·67
										Averages	40·73	13·33	22·47
G. 426	♀	XXII	*N* 1	28	84	56	14·6	27	131	104	38·95	13·69	22·68
G. 427	♂	XXII	*N* 1	28	84	56	17·7	34	191	157	39·85	13·28	22·07
										Averages	39·40	13·48	22·37
G. 428	♀	XXII	*N* 2	28	84	56	22·0	32	142	110	37·14	14·19	22·58
G. 429	♂	XXII	*N* 2	28	84	56	19·8	31	178	147	39·65	13·84	22·95
										Averages	38·38	14·01	22·76
G. 430	♀	XXII	*N* 2	28	84	56	16·5	29	136	107	35·85	14·56	22·70
G. 431	♂	XXII	*N* 2	28	84	56	18·5	33	193	160	38·83	14·28	23·34
										Averages	37·34	14·42	23·02

NOTE. The histological diagnosis in each rat is normal.

Table II.

All rats on normal diets	Average % H_2O	Average % Ca (wet bones)	Average % Ca (dry bones)
In darkness	40·03	13·30	22·13
In daylight + ultra-violet	38·88	13·49	22·06

In life there was no noticeable difference between the two groups. The growth curves of both males and females exceeded greatly the Donaldson



Rat G. 431. "Butter" Diet in Darkness. Age 84 days. Weight 160 grams. Rib.

Co. Cortical bone, thick and well calcified.
O. Physiological osteoid
Sp. Primary spongiosa. Trabeculae numerous, thick, straight and well calcified.
Z.P.C. Zone of provisional calcification well calcified.
H.C. Hypertrophic cartilage cells.
Z.C.P.C. Zone of proliferating cartilage cells.
R.C. Resting cartilage.
B.M. Bone marrow.

Diagnosis: Normal rib. Haem. and Eosin. Magn. × 40.
Note: The ribs of all the rats were similar to the one illustrated above.

curve of normal growth for the same period of life. The rats living in darkness grew as quickly and reached as high an average maximum weight as those kept in daylight and exposed daily to the mercury vapour quartz lamp.

At autopsy there were no gross differences between the two groups. The eyes were normal and the teeth light yellow and transparent. All the rats were well nourished with an abundant deposit of fat in the natural regions. The musculature was well developed and of good colour. The chest was well developed, and there was no macroscopical abnormality in the ribs or long bones.

Histologically, the bones were normal in every respect, those of the two groups showing practically no differences (see Table I and Pl. IV).

The chemical analysis of the hind legs, carried out by Aron's method [1910], showed a very close agreement between the two groups. In no case was the variation in the percentage of calcium and of water in the bones of these groups greater than that found normally among rats from the same litter (see Table I).

The individual (Table I) and average (Table II) results are remarkably close for the rats kept in darkness and those that were kept in the light and in addition were irradiated daily with the mercury vapour quartz lamp.

Summary.

Two litters of rats were placed on normal diets containing an optimal amount of calcium, phosphorus and the fat-soluble organic factor; in the diet of one litter this last was supplied by cod-liver oil, while for the other, two diets, one containing butter and the other cod-liver oil were used.

One-half of each litter was kept in the dark and the other in daylight with the addition of a daily irradiation for 20 minutes under a mercury vapour quartz lamp.

No appreciable difference was found between the growth of the two groups or histology and calcium content of the bones.

Conclusions.

Rats on a normal diet containing an optimal quantity of calcium, phosphorus, and fat-soluble organic factor, whether kept in darkness or in daylight supplemented by irradiation from a mercury vapour quartz lamp, grow equally well, and the development and calcification of their bones are normal.

We acknowledge our sincere thanks to Prof. C. J. Martin, for the privilege of carrying out the experiment at the Lister Institute.

REFERENCES.

Aron, H. and Sebauer, R. (1910). Abderhalden's Handbuch der biochemischen Arbeitsmethoden, **1**, 413.

McCollum, Simmonds, Shipley and Park (1921). *J. Biol. Chem.* **47**, 507.

Mellanby, E. (1921). *Experimental Rickets, Medical Research Council Special Report Series*, No. 61.

Raczynsky, J. (1912). *Compt. Rend. Assoc. Internat. Pédiatrie*, 308. Quoted by Park, *Phys. Rev.* 1923, **3**, 106.

XXXV. A STUDY OF THE RELATION OF THE QUANTITY OF FAT-SOLUBLE ORGANIC FACTOR IN THE DIET TO THE DEGREE OF CALCIFICATION OF THE BONES AND THE DEVELOPMENT OF EXPERIMENTAL RICKETS IN RATS.

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Introduction.

As a result of the pioneer work of Mellanby [1918, 1919, 1920, 1921] on experimental rickets in the dog, it was thought at first that this disease might prove to be a deficiency one, caused, like scurvy and beri-beri, by the lack of a single organic factor from the diet, in this case the fat-soluble organic factor called vitamin A or fat-soluble A. Later the work of Korenchevsky on experimental rickets in the rat tended to confirm Mellanby's early findings and conclusions. In his first publication Korenchevsky states that the deficiency of vitamin A in the diet of rats "produces in some rats a picture resembling slight rickets, and typical rickets in some young rats whose parents had been fed on a diet deficient in vitamin A during conception, pregnancy and lactation." Deficiency of fat-soluble A, or of some other unidentified organic factor very closely associated with it, is now recognised as one of the important factors in the etiology of experimental rickets in dogs and rats. But, to date, most investigators of this subject who believe in the dietetic origin of the disease are of the opinion that the deficiency of this organic factor cannot be the sole cause of the disease. Some authors [Hess and Shipley and collaborators, McCollum and Simmonds, 1921] are very emphatic that a deficiency of fat-soluble A alone in a diet otherwise complete does not produce experimental rickets in rats but merely interferes with osteogenesis and causes the development only of osteoporosis. Similar conclusions were reached by Zilva, Golding, Drummond and Coward [1921], Tozer [1921] and Mackay [1921] as the result of studies on the effects of vitamin A deficiency alone in the diet of pigs, guinea-pigs and cats.

In his first communication on the subject Korenchevsky [1921] refers to

the fat-soluble organic factor as vitamin A, which is the term usually applied to the growth-promoting, anti-xerophthalmic, fat-soluble, organic factor. But in a monograph [1922] recently published he refers to it as the "anti-rachitic organic factor," without admitting or denying its identity with the growth-promoting factor. The work of McCollum, Simmonds, Becker and Shipley [1922] on the nature of the fat-soluble organic factor tends to show that the fat-soluble growth-promoting or anti-xerophthalmic factor and the anti-rachitic or calcium-deposition-promoting factor are either two distinct parts of the one substance or two separate substances very similar in their distribution and many of their properties. In the present communication, since a coincident deficiency of both factors was of necessity dealt with in the investigation, the inclusive term fat-soluble organic factor or simply organic factor will be used when referring to both, and the words growth-promoting or anti-rachitic will be prefixed to the term where specific reference is made to one or other of the factors.

At the time this investigation was started most of the results referred to above had not yet been published. The study was undertaken with a view to determine whether, as Mellanby found in the case of the dog, a deficiency of the organic factor alone in the diet can also produce rickets in rats, and if so, whether a relation exists between the quantity of this factor in the diet and the degree of calcification or severity of the pathological lesion of the bones.

Experimental.

Part I.

In this investigation the rats employed were bred in the Institute from animals fed on fresh cow's milk, white bread, oats and bran, green cabbage leaves (all *ad libitum*) and occasionally some red meat. The milk and cabbage leaf were the main source of the fat-soluble organic factor in this diet. The experiments were carried out during the latter part of autumn and during the winter of 1921, so that the milk was "winter milk," and probably not very rich in the organic factor. Judging from the rapidity with which the offspring from rats kept on the above diet showed the effects of deficiency of the fat-soluble organic factor in their diet, one must conclude that their ante-natal (and post-natal up to the time of weaning) supply of that factor was perhaps adequate but by no means abundant. On this account the same experiments were repeated with rats whose parents had received a larger supply of the organic factor before impregnation and during pregnancy and lactation. This was supplied to the parent animals in the form of cod-liver oil. The results of those experiments are being reported in Part III of this communication. All the rats were kept in a well-lighted and ventilated room, but away from the action of direct sunlight. The windows, which were kept closed, were of very thick plate glass.

Diets. The synthetic diet adopted for the experiments consisted of the following ingredients:

"Casein" (commercial)	20 g.	Marmite	5 g.
Starch (wheat)	50 g.	Orange juice	5 cc.
Fat	15 g.	Distilled water	50 cc.
Salt mixture[1]	5 g.		

This was kneaded into the form of a very thick paste, kept in tightly stoppered glass jars in a refrigerator, and was made fresh about twice a week.

It has been shown by Shipley, Park, McCollum and Simmonds [1921] that a diet which contains 3 % of butter, provided that diet is adequate in all other respects, especially the quality and quantity of its protein and the quantity and proportion of its inorganic elements, supplies an amount of the fat-soluble organic factor sufficient to permit of normal growth, reproduction and normal calcification of the bones of rats. For the normal control diet of these experiments the synthetic diet mentioned above in which the entire amount of fat consisted of fresh butter was adopted. This diet contained 15 g. of butter per 100 g. of dry constituents or 10 % of the wet paste as administered to the rats. This diet will be referred to as diet *A*.

In order to grade the amount of organic factor, yet retain exactly the same proportion of the various constituents, an ingredient had to be used which contained a moderate amount of this factor and which could be purified of it. In this connection advantage was taken of the work of Osborne and Mendel [1915] and of some unpublished work of Zilva (to whom thanks is due for the suggestion) who found that unpurified commercial "casein," when it forms 18 % of a diet otherwise complete, contains sufficient fat-soluble organic factor to permit of moderate growth at first and later slight gradual increase or maintenance of weight over a long period. But the weight curve is never normal and reproduction occurs but rarely. However, the development of pathological lesions in the bones of animals on this diet has not been reported. By using varying proportions of unpurified and inactivated[2] casein, the content of organic factor in the synthetic diet mentioned was graded without altering the quantity of the ingredient. Five diets differing only in their content of

[1] This is normal salt mixture No. 185 employed by McCollum [1917], the only difference being that the magnesium sulphate used was not anhydrous so that the amount of this salt was altered in order to keep the proportion of magnesium the same.

Salt mixture 185.

Sodium chloride	43·5
Magnesium sulphate	137·4
Sodium acid phosphate	87·2
Potassium phosphate (basic) ...	239·8
Calcium diacid phosphate ...	135·7
Calcium lactate	326·8
Ferric citrate	29·6
	1000·0

[2] Inactivated casein free from traces of the growth-promoting fat-soluble organic factor detectable by the biological method of Zilva and Miura [1921] was obtained by the heating and oxidation method [see Hopkins, 1920].

unpurified commercial casein, and therefore in their content of organic factor, were thus made. The fat used for these diets was hardened cotton seed oil free from organic factor.

Diet	% unpurified casein	% inactivated casein
B	20	0
C	15	5
D	10	10
E	5	15
F	0	20

In this way a rough gradation of the amount of fat-soluble organic factor was obtained. The difference between diets *A* and *B* in respect of their content of organic factor is obviously great, though how great, it is difficult to say. It was impossible to obtain sufficient animals from the same litter to place on diets with increasing amounts of organic factor so as to breach this gap between diets *A* and *B*.

In all six diets used in these experiments vitamin B (antineuritic) was supplied in the form of marmite and vitamin C (antiscorbutic) in orange juice[1].

The ash of these diets is alkaline. McCollum, Simmonds, Shipley and Park [1922] came to the conclusion that the reaction of the diet did not affect its rickets-producing qualities, but in a recent communication, Zucker, Barnett and Johnson [1923], have shown that for the diets they used, an alkaline ash made the diet more rickets-producing, and that by changing the reaction of a moderately rickets-producing diet to acid, they could convert it into a non-rickets-producing diet. What the effect of changing the reaction of the above diets would have been cannot be stated in this communication.

The amount of calcium in the above diets (*B*, *C*, *D*, *E* and *F*) was estimated by the McCrudden method and was found to be 0·258 g. per 100 g. of the wet paste (as administered to the animals). The normal diet (*A*) contained 0·285 g. per 100 g. of wet paste, slightly more than the others on account of the calcium contained in the butter included in that diet. The amount of phosphorus (estimated by Neumann's method) was 0·548 g. per 100 g. of the wet paste, and was practically the same for all the diets. Estimated for the diets without water the percentage of calcium was approximately 0·4 g. and phosphorus 0·8 g. per 100 g. of diet. When compared with the amount of calcium in the diets employed by McCollum and his collaborators, 0·64 g. per 100 g. of "dry" diet (as administered to the rats) and by Hess and Sherman and their respective associates, 0·48 g. to 0·55 g. per 100 g. of dry diet, the amount of this element used in the above diets is apparently low. Also, instead of the more usual calcium-phosphorus ratio of about two to one (the proportion by weight of these two elements in normal bone) the proportion of calcium to phosphorus in the above diets was approximately

[1] Since these experiments were begun it has been shown by Osborne and Mendel [1922] that orange juice contains also a certain amount of the growth-promoting fat-soluble organic factor. In a few tests by the biological method this was confirmed but the effect of the amounts contained in the diets was found to be very weak. However, in subsequent experiments (Parts II and III) decitrated lemon juice, apparently free from fat-soluble organic factor, was employed as the source of vitamin C.

one to two. Korenchevsky [1922], who employed the same salt mixture and in the same proportion as used in these experiments when studying the effect on rats of complete deficiency of "vitamin A" in the diet, came to the conclusion that the calcium content of the diet was probably low, but in the absence of definite knowledge of what is the optimal amount, and since this must vary with the amount of anti-rachitic factor in the diet, he decided that in "experiments designed to test the influence of varying amounts of anti-rachitic factor, the most striking results may be expected to arise when the content of calcium in the diet is not excessive." Nevertheless, as will be seen later on, although the amount of calcium in the diet was apparently low, yet in the presence of an adequate amount of the fat-soluble organic factor (diet *A*) normal calcification of the bones occurred and rickets did not develop.

In order, therefore, to ascertain that a double deficiency (calcium and organic factor) was not being dealt with, other experiments were performed using diets with increased amounts of calcium, and the results of these are reported in Part II of this communication. It may be stated in short that those experiments demonstrated that the amount of calcium used here was adequate, but by no means excessive and probably not optimal.

Twelve series of rats, every series consisting of six animals from the same litter were employed. Unfortunately, it was not possible to have all the animals of a series of the same sex. In all quantitative work of this kind, for proper comparison, it is best to have all the representatives of a series of the same sex, for it has been the experience of most investigators that males and females differ in their growth on normal diet and in their response to deficiencies in the diet. The twelve litters varied in age from 24 to 41 days at the time special feeding was begun. In the tables, the families are numbered I to XII. All the series were not killed at the same time because of uncertainty about the optimal period necessary for the manifestation of a pathological lesion. Six series were killed after seven weeks of special feeding, and the remainder at intervals up to fourteen weeks, only one series being allowed to remain on the special diets for so long a period (see litter XII in Table I). From every series a representative was placed on every one of the six diets *A* to *F*. Food was given *ad libitum*. The animals were fed daily and the amount consumed the previous day was recorded. The rats on normal diet "*A*" ate more than those on the various deficient diets, and their ration kept increasing as they grew older. But those on the deficient diets *B* to *F* ate larger portions during the first five weeks of the experimental period.

The average amount eaten during the entire experimental period by all the animals on the various diets was as follows:

Diet	Average amount consumed daily g.
A	14·3
B	11·2
C	9·7
D	9·9
E	9·3
F	9·4

The above figures do not apply to individual rats because in these experiments three animals on the same diet were kept in one cage. In the experiments reported in Parts II and III the exact amount eaten by every rat is known because they were kept in separate cages.

Growth on the different diets.

On normal diet *A* the rats grew well, though not quite as well as animals whose parents had received cod liver oil during pregnancy and lactation. Towards the latter part of the experimental period two of the animals (R. 347 and R. 352), females, did not gain the normal weekly amount. In those cases this was correlated by loss of appetite which developed for some unknown reason. The rats on diets *B* and *C* for the most part continued to gain steadily though slowly, but did not reach the normal figure. In a few cases, after from seven to eight weeks on these diets, the rats ceased to gain and only maintained their weight. On diets *D* and *E* the animals grew slowly and most of them reached their maximum weight in from five to seven weeks. A few continued to gain a little every week until they were killed. Those that did cease to gain were able to maintain approximately their maximum weight. The animals on diet *F* (completely deficient in fat-soluble organic factor[1]) reached their maximum weight in from three-and-a-half to six weeks, depending upon the age and weight of the rat when it was placed on this diet. The older and heavier the rat when special feeding was begun, the more resistant to the deficient diet. All maintained approximately their maximum weight up to the time they were killed. Any indication of impending great loss of weight in one rat (loss of appetite or persistent slight decline of weight) was a signal to kill the whole series to which it belonged in order to avoid the complication of osteoporosis which starvation and loss of weight initiate in bones.

It can be seen in Chart 1 that the average maximum weight of the six series showed a gradation corresponding roughly with the gradation in the amount of organic factor in the diet. As mentioned before, the number of males and females in every group varied considerably so that the curve is not as striking as the indications are that it would have been, had all the animals been of the same sex. At *D* (Chart 1) this point is well illustrated. There is a rise in the curve although the content of the fat-soluble organic factor was less in this diet than in the one preceding it. But of the 12 animals on diet *D*, seven were male, while of the same number on diet *C*, only two were of that sex. Males generally reach a greater weight and gain more rapidly than females whether on normal or on deficient diets. This illustrates very well the fact that in all quantitative nutrition experiments, it is important for purposes of comparison to use animals of one sex in every series.

[1] Though diet *F* is referred to as being "completely" deficient in the organic factor, yet it is realised that undetectable traces were most likely present.

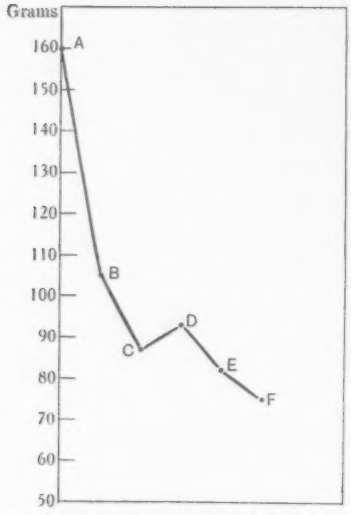

Chart 1. Average final weight of the rats on the different diets.

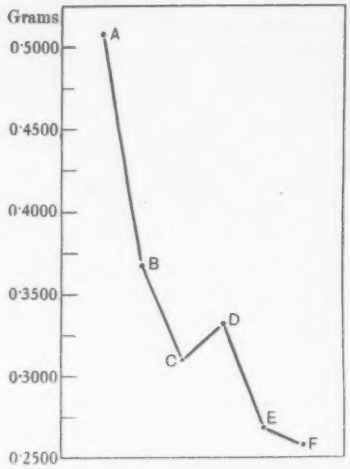

Chart 2. Average dry weight of bones (femur, tibia and fibula) of the rats on the different diets.

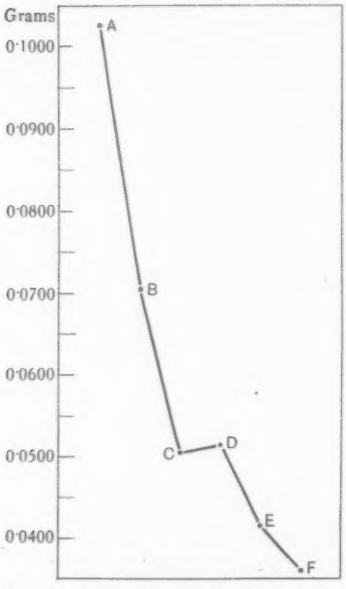

Chart 3. Average amount of calcium in the bones of the rats on the various diets.

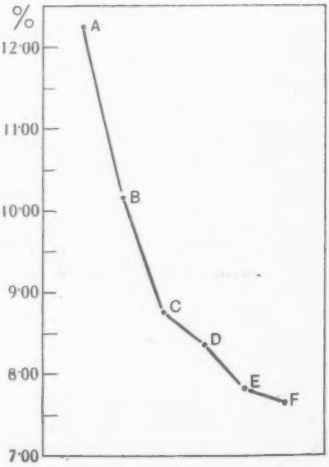

Chart 4. Average percentage of calcium per wet weight of bones.

Gross autopsy findings.

Although, as indicated in a previous part of this paper, the "store" of the fat-soluble organic factor in the rats used in these experiments was probably not very great at the time special feeding was begun, and although the calcium content of the experimental diets was probably not optimal, yet the control animals on diet *A* were, judging from gross appearances, quite normal. The fur was smooth and glossy, the eyes showed no abnormalities and the teeth in most cases presented the normal light yellow translucent appearance, though in a few rats there was a slight degree of opacity. The bodies seemed well proportioned, but no measurements were made. They were well nourished.

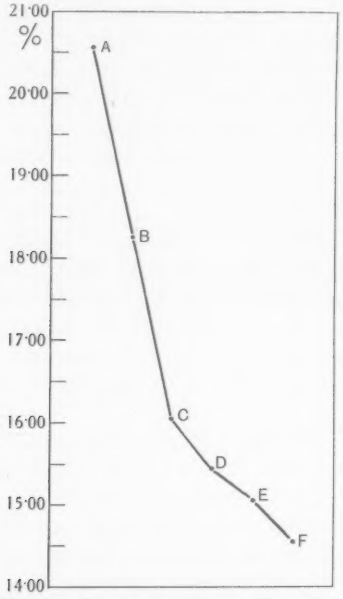

Chart 5. Average percentage of calcium per dry weight of bones.

There was an abundant deposition of fat in the normal regions, and the musculature was well developed and of good colour. The chest was full and rounded. The costal portion of the ribs was of the natural width and thickness for the size of the animals and, to the touch, appeared very well calcified. There were no deformities or fractures of any of the bones. The costochondral junction of the ribs was not enlarged, and the line of demarcation between cartilage and bone at the costochondral junctions was straight and very clearly defined. There was no epiphyseal enlargement of the long bones.

The animals on diet *B* differed grossly from the controls of the same litters on diet *A* in that they were smaller, lighter, and not as well nourished, though

better in all those respects than the rats on diets *C*, *D*, *E* and *F*, all poorer than *B* in their content of organic factor. The costal portion of the ribs was not as wide, as thick or as firm as that of the rats on diet *A*. In two rats (R. 367 and R. 369) several of the ribs showed a spontaneous fracture in the costal portion, but in no case was there any obvious deformity or enlargement at the costochondral junction of the ribs, or epiphyseal enlargement of the long bones. The shafts of the long bones were more readily broken than those of the normal rats.

The general appearance of the rats on diet *C* was similar to that of the animals on diet *B*, though they averaged a little smaller and lighter and were not quite so well nourished as the latter. The only striking difference was in the number of rats which had fractured ribs and the number of fractured ribs in those rats in which they occurred. The fractures occurred in four rats, affected from two to four ribs, and in one case there were several fractures in three ribs. In several rats there was slight enlargement of the costochondral junctions and the costochondral line was slightly curved and not quite sharply defined. The costal portion of the ribs and the shaft of the long bones were slightly more brittle than in those on diet *B*.

On diet *D* fractures were less frequent than in the rats on the foregoing diet. They occurred in two cases and were not multiple. There were no other striking differences between the animals on these two diets.

The rats on diet *E* presented a gross appearance similar in most respects to that of the rats on diet *F* to be described in detail below. They were slightly heavier, however, and a shade better nourished than those on diet *F*, but fractures occurred in the same number of cases (50 %), affected more ribs and were more often multiple.

The appearance of the rats on diet *F* was strikingly different from that of the animals on diet *A* from the same litters. They were very much smaller and lighter (about half or less) and were poorly nourished. The coat was rough and dull, and the front teeth in all cases were white and very opaque. This opacity of the teeth was always one of the first signs to develop as a result of deficient fat-soluble organic factor in the diet. All the animals on the above diets, with the exception of *A*, showed this to a more or less marked degree at the time they were killed. In no case did xerophthalmia develop. The chest in most of these animals was poorly developed, presenting the appearance of a tall cone with narrow base and, in many cases, sunken wall. Only in a few instances (rats R. 411, 413 and 415) were the costochondral junctions of the ribs greatly enlarged. In some there was no enlargement at all, while in the remainder this was only from slight to moderate. In 50 % of the animals on this diet fractures were present in many ribs, and in some the fractures were multiple. In both of these respects the condition was not quite as bad in the rats on diet *F* as in those on diet *E*. In the rats on both of these diets (*E* and *F*) the fracture was surrounded by a large, white, rather firm callus. The bony portion of the ribs varied in thickness and width

in the different animals, being very narrow and thin in some, and of moderate width and thickness in others, but in no case were they even approximately normal, with the exception of rat 418, which was placed on the deficient diet when aged 41 days and weighing 60 g. The costal portion of the ribs could be bent and broken with ease compared with the normal, and the same held true in most instances for the shaft of the long bones. In a few rats the lower epiphysis of the radius was obviously enlarged. This was also the case in some of the animals on diet *E*. In no case was there any evident deformity or fracture of the shaft of the long bones which could be recognised at autopsy. Histologically, however, one infraction of a radius from a rat on diet *E* was discovered.

The internal organs of the rats on the various diets showed no obvious gross pathology. In a few rats on diets *E* and *F* slight enlargement of the spleen was noted.

Histology.

Technique. From every animal, three or more ribs, one radius and one humerus were taken for histological examination. These were first fixed in formalin (10 % in normal saline) for 48 hours, and then subjected to slow decalcification in Mueller's fluid. In a few cases it was possible to cut the bones without previous decalcification. The latter, when sectioned, were treated with silver nitrate (the von Kossa method) and counterstained with alum, carmine and eosin. The sections of the bones which were partly decalcified in Mueller's fluid were stained with haematoxylin and eosin.

The microscopical appearance of the ribs and long bones of the animals on diet *A* was in most cases quite normal within the limits of natural variation. But in two rats (R. 347 and R. 352) a slight degree of osteoporosis complicated the picture. Both of these animals were gaining very little weight during the last two weeks of the experimental period. (For illustration of normal rib see Pl. V, figs. 1 and 2.)

In no rat on the various deficient diets (*B*, *C*, *D*, *E* and *F*) was the histology of the bones absolutely normal. But in a number of rats on the less deficient diets, especially diet *B*, the microscopical appearance of the bones was found to be nearly normal, and they are classified as such under the heading of diagnosis in Table I. These differed from the normal in that the cortex was thinner, the bone marrow cavity wider and the trabeculae of the primary spongiosa were fewer, thinner and less regular in their arrangement. But there were no cartilagenous changes and cortex, trabeculae and provisional zone of calcification were normally calcified. Thus a slight degree of osteoporosis complicated the otherwise normal picture.

Where a definite increase of cortical osteoid was present, without any abnormal changes in the cartilage, a diagnosis of osteomalacia was made, the degree varying with the amount of osteoid (see Plate V, fig. 4). It is realised that morphologically this cannot be distinguished from some cases of very late rickets, and that some authors now use as the only criterion for the

Table I.

No. of rat	Sex	No. of litter	Diet	Age (in days) Initial	Final	Days on diet	Weight (g.) Initial	Final	Gain in g.	In bones % H_2O	% Ca wet	% Ca dry	Histological diagnosis
R. 347	♀	I	*A*	32	81	49	38	110	72	43·66	11·60	20·33	Nearly normal (very slight osteoporosis)
R. 350	♂♀	II	*A*	34	83	49	47	177	130	40·95	12·52	21·20	Normal
R. 351	♂♀	III	*A*	30	79	49	41	177	136	40·53	12·00	20·18	,,
R. 354	♂♀	IV	*A*	26	74	48	23	125	102	42·49	11·03	19·31	,,
R. 356	♂♂♀	V	*A*	27	75	48	35	162	127	44·82	11·39	20·65	,,
R. 357	♂♂♂	VI	*A*	24	72	48	21	137	116	41·83	12·03	20·69	,,
R. 348	♂♂♀	VII	*A*	32	94	62	48	183	135	40·73	12·33	21·00	,,
R. 352	♀	VIII	*A*	33	94	61	42	113	71	41·29	12·10	20·61	Nearly normal (very slight osteoporosis)
R. 353	♂♀	IX	*A*	25	86	61	22	165	143	41·96	11·83	20·39	Normal
R. 355	♂♀	X	*A*	26	88	62	25	126	101	40·40	11·86	19·91	,,
R. 349	♂♂	XI	*A*	34	122	88	47	200	153	33·96	14·19	21·32	,,
R. 358	♂	XII	*A*	41	137	96	61	247	186	33·94	13·95	21·56	,,
									Averages	40·54	12·23	20·59	
R. 359	♀	I	*B*	32	81	49	50	104	54	42·09	10·47	18·05	Nearly normal
R. 362	♀♀	II	*B*	34	83	49	42	93	51	43·96	10·91	19·47	,, ,,
R. 363	♀♀	III	*B*	30	79	49	33	103	70	45·92	10·34	19·17	,, ,,
R. 366	♀	IV	*B*	26	—	—	—	—	—	—	—	—	Died. Enteritis. Osteoporosis
R. 368	♂	V	*B*	27	75	48	38	124	86	51·49	7·92	16·34	Moderate osteoporosis and very slight osteomalacia
R. 369	♀	VI	*B*	24	72	48	25	81	56	52·25	7·40	14·77	Moderate osteoporosis, slight osteomalacia
R. 360	♂♂	VII	*B*	32	94	62	44	148	104	43·15	10·85	19·09	Nearly normal
R. 364	♂♂	VIII	*B*	33	94	61	43	125	82	43·62	10·28	18·44	Very slight osteomalacia
R. 365	♀♀	IX	*B*	25	—	—	—	—	—	—	—	—	Died. Enteritis. Severe osteoporosis
R. 367	♀	X	*B*	26	88	62	27	91	64	45·93	9·49	17·56	Slight osteomalacia
R. 361	♀	XI	*B*	34	122	88	42	96	54	41·13	11·15	18·95	Nearly normal
R. 370	♀	XII	*B*	41	137	96	58	133	75	37·88	12·95	20·58	,, ,,
									Averages	44·74	10·17	18·24	
R. 371	♂	I	*C*	32	81	49	48	93	45	44·40	8·83	15·89	Slight osteomalacia
R. 374	♂♀	II	*C*	34	83	49	46	84	38	51·34	8·04	16·53	Moderate osteomalacia and slight osteoporosis
R. 375	♀	III	*C*	30	79	49	43	93	50	42·99	9·13	16·00	Very slight osteomalacia
R. 378	♀	IV	*C*	26	74	48	23	77	54	50·05	6·54	13·10	Moderate rickets
R. 380	♀	V	*C*	27	75	48	22	63	41	50·06	8·32	16·66	Very slight osteomalacia and osteoporosis
R. 381	♀	VI	*C*	24	72	48	22	70	48	53·25	6·58	14·09	Slight rickets and moderate osteoporosis
R. 372	♂♂	VII	*C*	32	94	62	43	108	65	36·50	10·62	17·76	,, osteomalacia
R. 376	♀	VIII	*C*	33	94	61	36	82	46	41·09	9·74	16·59	Moderate osteoporosis and slight osteomalacia
R. 377	♀	IX	*C*	25	86	61	25	65	40	48·33	6·28	12·16	,, ,, ,,
R. 379	♀	X	*C*	26	88	62	26	82	56	46·18	8·98	16·74	Very slight osteomalacia
R. 373	♀	XI	*C*	34	122	88	48	108	60	38·04	11·05	17·84	Nearly normal
R. 382	♀	XII	*C*	41	137	96	54	127	73	40·74	11·25	19·00	,, ,,
									Averages	45·26	8·78	16·03	
R. 383	♂	I	*D*	32	81	49	43	105	62	48·38	7·63	14·79	Moderate rickets and osteoporosis

R. 383	♂	I	*D*	32	81	49	43	105	62	48·38	7·63	14·79	Moderate rickets and osteoporosis
R. 386	♂♀	II	*D*	34	83	49	50	110	60	44·09	9·55	16·88	Slight osteomalacia and osteoporosis
R. 387	♀	III	*D*	30	79	49	38	84	46	42·66	9·86	17·20	Moderate osteoporosis
R. 390	♀	IV	*D*	26	74	48	25	62	37	53·73	5·60	12·15	„ rickets
R. 392	♂♀	V	*D*	27	75	48	33	80	47	50·75	8·21	16·68	Slight osteoporosis
R. 393	♀	VI	*D*	24	72	48	23	69	46	45·23	7·15	14·37	Moderate rickets and osteoporosis
R. 384	♀	VII	*D*	32	94	62	47	80	33	40·97	8·31	13·92	Very slight osteomalacia and osteoporosis
R. 388	♂♀	VIII	*D*	33	94	61	40	104	64	45·38	8·32	15·24	„ rickets and moderate osteoporosis
R. 389	♀	IX	*D*	25	86	61	21	48	27	49·77	6·12	12·19	Moderate rickets and slight osteoporosis
R. 391	♂	X	*D*	26	88	62	30	96	66	48·46	7·64	14·83	„ osteomalacia
R. 385	♂	XI	*D*	34	122	88	47	150	103	39·95	11·56	19·93	Nearly normal
R. 394	♂	XII	*D*	41	137	96	60	145	85	41·01	10·36	17·56	Slight osteoporosis
									Averages	45·86	8·36	15·47	
R. 395	♂	I	*E*	32	81	49	53	84	31	44·84	7·62	13·82	Moderate osteomalacia
R. 398	♀	II	*E*	34	83	49	40	7±	34	48·95	9·01	17·58	Slight osteomalacia
R. 399	♂♀	III	*E*	30	79	49	49	111	62	48·59	8·25	16·08	Moderate osteomalacia and slight osteoporosis
R. 402	♀	IV	*E*	26	74	48	25	52	27	54·46	4·88	10·69	„ rickets and osteoporosis
R. 404	♂♀	V	*E*	27	75	48	33	82	49	52·67	6·22	13·14	Slight osteomalacia
R. 405	♀	VI	*E*	24	72	48	20	49	29	55·70	4·98	11·58	Severe rickets
R. 396	♂	VII	*E*	32	94	62	50	101	51	44·40	9·10	16·51	Moderate osteomalacia
R. 400	♂	VIII	*E*	33	94	61	42	82	40	49·27	7·89	15·73	„ rickets
R. 401	♂♀	IX	*E*	25	86	61	28	68	40	47·38	6·59	12·52	„ „
R. 403	♀	X	*E*	26	—	—	—	—	—	—	—	—	Killed and eaten
R. 397	♂	XI	*E*	34	122	88	42	99	57	47·19	9·25	17·52	Slight osteoporosis
R. 406	♀	XII	*E*	41	137	96	57	120	63	41·69	12·20	20·92	Nearly normal
									Averages	48·65	7·81	15·09	
R. 407	♂	I	*F*	32	81	49	43	66	23	44·37	6·89	12·44	Very slight rickets and osteroporosis
R. 410	♀	II	*F*	34	83	49	38	75	37	45·63	9·50	17·37	Slight osteoporosis
R. 411	♂	III	*F*	30	79	49	44	109	65	48·85	7·52	14·69	Moderate rickets
R. 414	?	IV	*F*	26	—	—	—	—	—	—	—	—	Killed and eaten
R. 416	♀	V	*F*	27	75	48	33	63	30	48·80	6·33	12·37	Slight osteomalacia and moderate osteoporosis
R. 417	♀	VI	*F*	24	72	48	23	54	31	46·41	6·85	12·87	Very slight rickets and moderate osteoporosis
R. 408	♀	VII	*F*	32	62	30	—	—	—	—	—	—	Slight osteomalacia and moderate osteoporosis
R. 412	♀	VIII	*F*	33	94	61	49	62	13	44·25	7·46	13·11	Moderate osteoporosis
R. 413	♀	IX	*F*	25	86	61	27	58	31	53·27	5·80	12·44	Severe rickets
R. 415	♀	X	*F*	26	88	62	25	62	37	52·77	7·15	15·10	Moderate rickets
R. 409	♂	XI	*F*	34	122	88	43	77	34	49·05	8·19	16·00	Slight osteoporosis and very slight osteomalacia
R. 418	♂	XII	*F*	41	137	96	60	125	65	44·06	10·85	19·28	Slight osteoporosis
									Averages	47·75	7·65	14·56	

diagnosis of rickets, a definite increase in the amount of osteoid tissue regardless of the presence or absence of changes in the cartilage.

If, in addition to the increase of endosteal, periosteal and peritrabecular osteoid, pathological changes were also present in the epiphyseal cartilage or in that at the costochondral junctions, then a diagnosis of rickets was made, the degree depending "arbitrarily" upon the severity of the lesions. These lesions were the increased proliferation, disorganisation and imperfect calcification of the cartilage at the zone of provisional calcification, the presence and character of a rachitic metaphysis and the amount of osteoid. In some cases there were fractures, the callus of which consisted mainly of osteoid, poorly calcified or uncalcified cartilage and fibrous connective tissue (see Pl. VI, figs. 5–8 for varying degrees of rickets).

Both osteomalacia and rickets occurred in the rats on diets *C*, *D*, *E* and *F*. Both were in many cases complicated by some degree of osteoporosis and this is indicated under the heading of diagnosis in Table I.

The following table (Table II) shows a comparison of the number of normal, nearly normal, and pathological cases which occurred on the various diets. In this table they are classified according to the predominating lesion present, and in the case of osteomalacia and of rickets regard is taken of the severity of the condition.

Table II.

					Osteomalacia			Rickets			
Diet	Total No. of Rats	Normal	Nearly normal	Osteo-porosis	Very slight	Slight	Moderate	Very slight	Slight	Moderate	Severe
A	12	10	2	0	0	0	0	0	0	0	0
B	12	0	6	4	1	1	0	0	0	0	0
C	12	0	2	2	3	2	1	0	1	1	0
D	12	0	1	3	1	1	1	1	0	4	0
E	11	0	1	1	0	2	3	0	0	3	1
F	11	0	0	5	0	1	0	2	0	2	1

It is seen in the above table that rickets and osteomalacia were most frequent in the rats on diets *D*, *E* and *F* and that the two severe cases of rickets occurred in rats R. 405 and R. 413, on diets *E* and *F* respectively.

In the rats on diet *F*, pure osteoporosis occurred more frequently than in those on the other diets. Most investigators who have studied the pathology of the bones of rats on a diet completely deficient in fat-soluble organic factor have either killed the rats after they had been on the diet for a very long time, when, in many instances, they were losing weight very rapidly, or studied the bones of rats that had died after a long period on such a diet. It is probable that this fact accounts for the divergent results obtained and consequently for the difference of opinion held by investigators of the effect of deprivation of the fat-soluble organic factor alone from the diet of rats.

Analysis of bones for calcium content.

The calcium content of the bones was estimated by Aron's method [Aron and Sebauer, 1910]. Femur, tibia and fibula were chosen for analysis. These

were mechanically cleaned of all soft tissue with the exception of the ligaments round, and the cartilage and ligaments within, the knee-joint. The patella was removed. Each hind leg was analysed separately, but the percentage of calcium and of water corresponded so closely in the duplicate estimations that in Table I only the average of the two is given (see also Charts 2–5). The following table (Table III) gives the average figures for all the rats on the various diets.

Table III.

Diet	% Ca per wet weight of bones	% Ca per dry weight of bones	Deficiency of Ca as % of normal		% H_2O in bones	Excess of H_2O as % of normal
			Wet bones	Dry bones		
A	12·23	20·59	—	—	40·54	—
B	10·17	18·24	16·8	11·4	44·74	10·3
C	8·78	16·03	28·2	22·1	45·26	11·6
D	8·36	15·47	31·7	24·8	45·86	13·1
E	7·81	15·09	36·2	26·7	48·65	20·0
F	7·65	14·56	37·5	29·3	47·75	17·7

Table III shows a striking difference between the percentage of calcium in the bones of rats on diet *A* and that of the remaining five diets which differ from it only in the fact that their content of fat-soluble organic factor is less. In this table also a definite gradation of the average percentage of calcium in the bones is noted which corresponds with the gradation in the amount of fat-soluble organic factor in the diets (see Chart 5). The differences are not nearly as great between the remaining diets in respect either of calcium content of the bones or organic factor content of the diets. But there is as much or more difference between the calcium content of the bones of rats on diets *F* and *B* as between those on diets *B* and *A*. However, in the individual series (see Table I) this regular gradation is not always seen. The fact that the animals of a series were not always of the same sex probably interfered with the regularity of the gradation in calcium content, as it did in the case of the growth curves mentioned above.

When the amount of fat-soluble organic factor in the diet is adequate, the percentage of calcium deposited in the bones of rats of approximately the same age is remarkably uniform regardless of the weight of the animals, which may vary within fairly wide limits. At the age of about four months a rat on normal diet reaches its maximum degree of calcification.

On the other hand the rats on diets deficient in fat-soluble organic factor, especially those on diets *E* and *F*, showed very great variation in the calcium content of the bones, without any very definite relation to their final weight but with some relation to the age at which they were put on the deficient diets. The older and heavier the animal at the time it was put on the deficient diet the more likely it was to have a relatively high calcium content especially if not kept on the diet very long. (Compare rat R. 418 with R. 409 both on diet *F*, and R. 406 with R. 397 both on diet *E*.) The corresponding rats from

the same litters on the other diets more rich in organic factor showed less difference in their calcium content, despite the difference in the initial age, and, on the normal diet (*A*), rat R. 349 actually had a higher percentage of calcium in its bones than rat R. 358 despite the fact that the initial and final age of the former was less than that of the latter (see Table I).

The water content of the bones of rats on diet *A* was from 33 % to 42 % of the wet weight of the bones. In the animals on diet *F* the water content of the bones varied from 44 % to 53 %. The rats on diets *B*, *C*, *D* and *E* also showed a definite increase in the percentage of water. The water content of the bones increased as the amount of organic factor in the diet decreased (see Table II).

Summary and Discussion.

A study has been made of the relation of the quantity of fat-soluble organic factor in a diet to the degree of calcium deposition in the bones and to the development of experimental rickets in rats kept away from direct sunlight.

By grading the amount of fat-soluble organic factor in the diet and leaving all other constituents constant, there resulted a corresponding gradation in the percentage of calcium deposited in the bones. It is, therefore, evident that the organic factor plays an important role in the mechanism for the deposition of calcium in bones, but how it acts is not elucidated by this investigation.

Korenchevsky found that with his "—A diet," which corresponds to diet *F* described above, he produced in some young rats a picture resembling rickets. This is confirmed by the results of the above investigation. What is more, it is shown that complete absence of the fat-soluble organic factor from the synthetic diet used is not necessary in order to initiate in the bones of some young rats changes which bear a definite resemblance to rickets or osteomalacia. In fact more cases of rickets and osteomalacia occurred in the rats on diets *D* and *E* than on diet *F*, which was the most deficient in the organic factor. This is perhaps best explained by the fact that on the latter diet complicating osteoporosis was more frequent and more severe, and this masked or prevented manifestation of the rachitic lesion.

Whenever the effect of the diminution or complete absence of only one of several variable factors is being investigated it is necessary that the others should be present in at least optimal quantity if not in excess. The three substances in the diet which, so far, have been considered to bear an important relation to the development of experimental rickets in rats are fat-soluble organic factor, calcium and phosphorus. Compared with the percentage used by other investigators the quantity of phosphorus in the above diets was certainly optimal. But the same cannot be said of calcium, for it was present in appreciably lower amounts than that recommended as the optimum by other investigators. McCollum and his collaborators, for the type

Table IV.

No. of rat	Sex	No. of litter	Diet	Age (in days) Initial	Age (in days) Final	Days on diet	Average amount of food eaten daily	Weight (g.) Initial	Weight (g.) Final	Gain in g.	In bones % H_2O	In bones % Ca wet	In bones % Ca dry	Histological diagnosis
G. 370	♂	XIII	*F*	27	83	56	10·4	42	94	52	46·95	7·83	14·80	Very slight rickets and slight osteoporosis
G. 371	♂	XIII	*F*	27	83	56	10·4	39	79	40	53·25	6·43	13·69	Moderate rickets
										Averages	50·10	7·13	14·24	
G. 372	♂	XIII	*F* 1	27	83	56	10·2	42	87	45	51·83	7·59	15·77	Very slight rickets and osteoporosis
G. 373	♂	XIII	*F* 1	27	83	56	10·2	38	90	52	51·64	6·92	14·32	Moderate rickets
										Averages	51·73	7·25	15·04	
G. 374	♂	XIII	*F* 2	27	83	56	11·6	42	95	53	48·10	9·81	18·90	Moderate osteoporosis
G. 375	♂	XIII	*F* 2	27	83	56	11·6	34	112	78	45·94	9·25	17·11	Slight osteoporosis
										Averages	47·02	9·53	18·00	
G. 376	♂	XIV	*F*	23	79	56	9·7	41	71	30	50·05	6·78	13·71	Slight rickets
G. 378	♀	XIV	*F*	23	79	56	9·7	43	76	33	48·80	6·77	13·23	Moderate rickets
										Averages	49·42	6·77	13·47	
G. 379	♀	XIV	*F* 1	23	79	56	9·4	41	80	39	44·14	8·12	14·60	Slight rickets
G. 380	♀	XIV	*F* 1	23	79	56	9·4	39	87	48	48·97	7·58	14·87	Moderate rickets
										Averages	46·69	7·85	14·73	
G. 381	♂	XIV	*F* 2	23	79	56	10·1	41	100	59	44·12	8·20	14·68	Slight rickets
G. 382	♀	XIV	*F* 2	23	79	56	10·1	36	82	46	45·95	8·30	15·36	Moderate osteomalacia
										Averages	45·03	8·25	15·02	
G. 339	♀	XV	*F*	27	78	51	9·4	31	56	25	54·30	6·53	14·28	Moderate osteomalacia
G. 340	♀	XV	*F*	27	68	41	9·4	33	54	21	48·20	5·72	11·50	Slight rickets and osteoporosis
G. 341	♂	XV	*F*	27	78	51	10·5	38	93	55	48·19	7·73	14·92	Slight osteoporosis
										Averages	50·23	6·66	13·56	
G. 342	♀	XV	*F* 1	27	78	51	11·7	34	87	53	43·83	8·90	15·84	Slight osteomalacia
G. 343	♀	XV	*F* 1	27	68	41	11·7	31	65	24	51·34	7·32	15·16	„ rickets
G. 344	♂	XV	*F* 1	27	78	51	14·7	35	89	54	45·05	8·19	14·90	„ osteomalacia
										Averages	46·74	8·13	15·30	

of diets used by them, recommend 0·64 g. per 100 g. of dry substance as the optimum for calcium, while in diets *A* to *F* used in this investigation the amount of calcium was 0·258 g. per 100 g. of wet paste or 0·42 g. per 100 g. of diet without water or orange juice. How close this quantity of calcium is to the optimal amount for the type of diet used cannot be stated, and without definite knowledge of the amount of this element absorbed from the diet by rats, cannot be correctly estimated. Therefore, an investigation was made of the effect of increasing the quantity of calcium in the diet most deficient in organic factor (diet *F*) used in the above experiments, and the result is reported in Part II of this communication.

Part II.

The effect of increasing the calcium content of diet F.

In order to ascertain whether, in the experiments described in Part I, one was dealing with a deficiency of calcium as well as of fat-soluble organic factor, the amount of calcium was increased in diet *F* (the diet most deficient in organic factor) and the effect on the bones of rats of the administration of this diet enriched in calcium content was investigated. To diet *F*, as used in the experiments of Part I, calcium carbonate was added so that two new diets were made, one with double and the other with four times the quantity of calcium contained in diet *F*, which will be referred to as diet *F* 1 and *F* 2 respectively.

Diet	Calcium per 100 g. of wet diet	Phosphorus per 100 g. of wet diet	Proportion of Ca to P
F	0·258	0·538	0·479 : 1
F 1	0·538	0·538	1 : 1
F 2	1·076	0·538	2 : 1

Experiments.

Series I. Of a litter of six rats similar in origin to those used in Part I, two were placed on diet *F* and the same number on each of the new diets, *F* 1 and *F* 2, containing increased amounts of calcium. They were kept on these diets for eight weeks. The results of chemical analysis and histological examination of the bones are given in Table IV.

Series II. Another litter of six rats was divided equally between the three diets. The results are summarised in Table IV.

Series III. Three rats of a litter of six were placed on diet *F* and three on *F* 1. The details of the experiment are given in Table IV.

The following is a comparison of the *average* percentage of water and of calcium in the bones of all the rats on the three diets.

Table V.

Diet	Average % Ca in wet bones	Average % Ca in dry bones	Average % H_2O in bones
F	6·95	13·85	50·39
F 1	7·54	14·89	49·21
F 2	8·89	16·51	46·02

An appreciable increase is noted in the average percentage of calcium per wet and dry weight of the bones, and a decrease in the average percentage of water in the bones of rats on diets *F* 1 and *F* 2 as compared with *F*. The highest average calcium content and the lowest water content was in the rats on diet *F* 2 which contained the greatest amount of calcium. But the differences between the three groups were not very great, and in all the rats the calcium content of the bones was very far below the normal, which, for rats of that age, should be about 13 % per wet weight and 21 % per dry weight. The water content of the bones should be about 38 % for normal rats of that age. Therefore, in all the rats of the three series the percentage of water in the bones is definitely increased.

The only gross differences between the rats on diets *F* 1 and *F* 2 and those on diet *F* were that the former averaged slightly larger and heavier (though their nourishment was no better) and fractures occurred less frequently. In the ribs of only one case on diet *F* 2 did fractures occur, in three on diet *F* 1 and in five on diet *F*.

Histologically, the differences between the bones of the three types of rats were not very great. In the main, the bones resembled those described for diet *F* in Part I of this communication. However, although the three types were distinctly abnormal, the bone lesions varying from pure osteoporosis to slight rickets (see Table IV) yet, in the main, the pathological condition was most severe in the rats on diet *F* and least in those on diet *F* 2. There was but little to choose between those on diets *F* and *F* 1 although the latter contained double the quantity of calcium, an amount practically equal to that considered optimal by McCollum and his collaborators.

Summary of Part II.

Although the increase of the amount of calcium in diet *F* so that it was optimal (diet *F* 1) and even excessive (diet *F* 2) resulted in a corresponding but not very great average increase of calcium deposit in the bones, yet it did not prevent the development of pathological lesions in the bones similar to those described for diet *F* in Part I of this investigation. From the above results one must conclude that the amount of calcium in diet *F* was adequate to protect against the development of pathological changes in the bones provided the other constituents of that diet were present in adequate amounts. Therefore, since the only known dietetic deficiency in diets *F*, *F* 1 and *F* 2 was the fat-soluble organic factor, the above results serve as further proof that a diet deficient in the fat-soluble organic factor alone and otherwise complete, when administered to rats without exposing them to direct sunlight or any artificial substitutes for it, can initiate in the bones of some young rats a pathological condition resembling rickets or osteomalacia.

Part III.

The effect of increasing the pre-experimental store of fat-soluble organic factor upon the degree of calcification and development of the bones of rats on diets similar to those used in Part I.

Coward, Lush and Palmer [1923], have shown that a rat stores an appreciable amount of the growth-promoting anti-xerophthalmic organic factor of its diet in at least one of its organs, the liver. The storage of this factor in the body of the rat has also been inferred from the indirect proof furnished by studies of the growth of rats from different litters on a diet completely deficient in the fat-soluble growth-promoting factor. The tremendous variation in the growth curves led to the conclusion that the store of this factor in the young depends upon the amount of it in the mother's milk and therefore, in the diet of the mother. It was suggested in Part I of this communication that the store of fat-soluble organic factor in the rats employed in those experiments was not very great at the time special feeding was begun, and that it was possibly a combined effect of slight deficiency of this factor during the pre-experimental period and the greater deficiency of it during the period of special feeding which was responsible for the poor calcification and the pathology of the bones.

Having determined (Part II) that the mere increase of the amount of calcium in the most deficient diet (*F*) employed in the experiments of Part I did not prevent the development of pathological changes in the bones, it was considered important to investigate the influence of the pre-natal and post-natal (up to the time the animals were put on the special diets) supply of the fat-soluble organic factor upon the effects produced by the administration of diets *B*, *C*, *D*, *E* and *F* described in Part I.

In order to do this, litters were bred from rats to whose general diet (similar to that of the parent rats described in Part I) was added a paste containing cod-liver oil as the extra source of the fat-soluble organic factor. This will be referred to as the "cod-liver oil breeding diet" and was constituted as follows:

Cod-liver oil breeding diet:

Commercial "casein"	20 g.	Tricalcium phosphate	4·8 g.
Starch	60 g.	Calcium lactate	0·3 g.
Cod-liver oil	5 g.	Potassium phosphate	0·6 g.
Yeast	6 g.	Water	67 cc.
Sodium chloride	0·3 g.		

Of this paste about 10 g. was given daily to every breeding rat prior to and during pregnancy, and the ration was increased up to about 25 g. during the period of lactation. As a result, these breeding rats, both male and female, were in excellent condition, reproduction occurred frequently, and the effect

on the young was very striking. At the age of from 18 to 21 days most of these young rats were as heavy as animals aged 25 to 28 days whose parents were on the same general diet but without the addition of cod-liver oil breeding paste. The young probably ate some of this special breeding paste for four or five days prior to the time they were put on experiment. With four litters of these specially bred rats, every litter consisting of seven females, the experiments described in Part I were repeated.

In order to give the extreme test to the prophylactic action of cod-liver oil in the parents' diet on the prevention of bone pathology in the young placed after weaning on the various diets deficient in organic factor (*B–F*), the rats were put on these special diets at the age of three weeks, much earlier than in the experiments of Part I. In the latter, the rats varied from 24 to 41 days, the average age at the time they were put on the special diets being 30 days.

The only changes were the substitution of decitrated lemon juice for the orange juice in all the diets, and the addition of a seventh diet, another normal control diet, containing 5 g. of cod-liver oil which replaced 5 g. of cotton-seed oil in diet *B*, but was otherwise similarly constituted. This diet will be referred to as diet "*AA*."

Diet "AA":

Starch (wheat)	50 g.	Salt mixture (No. 185)	5 g.
Unpurified "casein" (commercial)	20 g.	Lemon juice (decitrated)	5 cc.
Cod-liver oil	5 g.	Marmite	5 g.
Hardened cotton-seed oil	10 g.	Water (distilled)	50 cc.

From every litter a rat was placed on every one of the seven diets. The four litters were three weeks old at the time they were placed on the special diets, and they were all kept on them for nine weeks. This is a slightly longer period than was used in the experiments of Part I, but these rats felt the deficiency of the organic factor in the various diets so much less than those of the first investigation, that it was deemed advisable to allow them a longer period on the special diets in order to give the deficiency of the organic factor enough time to act.

The control rats on normal diets *AA* and *A* grew very well indeed, those on diet *A*, which contained butter, growing as well and looking as normal as those on diet *AA* which contained cod-liver oil.

The effect of the good reserve store of the fat-soluble organic factor was very noticeable, for all the rats on the various deficient diets, even those on the most deficient (diet *F*), gained weight more quickly, continued to gain for a longer period, reached a higher maximum weight and appeared more nearly normal than the rats on the corresponding diets in the experiments described in Part I. However, there was a definite difference between the growth of the rats on diets *AA* and *A* and that of the rats on the first deficient diet (*B*). But, unlike the results in the first investigation, very little difference

was noted in the growth and general condition of the rats on the five deficient diets. The gradation in the average maximum weight of the rats corresponding with the amount of organic factor in the diet which was noted in Part I did not manifest itself at the end of the experimental period. In fact, in a few instances the rats on diet *F* reached a maximum weight equal to that of some on diet *B*. The average final weight and average maximum gain differed but little in the rats on the five deficient diets but all were from 20 to 30 % less than the normal in those respects (see Table VI).

Macroscopical findings.

At autopsy, the rats on diets *AA* and *A* presented no gross abnormalities and were better nourished and developed than the rats on diet *A* in the first investigation. The butter used for diet *A* in the experiments of Part I was winter butter whereas summer butter was used in this investigation. The rats on the diet containing summer butter were similar in all respects to those receiving cod-liver oil in their food. Of course it should be noted that relatively much more butter than cod-liver oil was used, but the amount of cod-liver oil in diet *AA* was optimal.

The rats on the various deficient diets were not as large and not nearly as well nourished as the ones on the normal diets. Those on diets *B* and *C* from the same litters differed but little from one another, but on each of those diets the representatives from the four different litters did vary somewhat in their gross appearance. The teeth of some of these rats were slightly opaque, and the bony portions of the ribs and the shafts of the long bones were thinner and more brittle than normal. In one rat on diet *B* (G. 49) and one on diet *C* (G. 50) both from the same litter, there was very slight enlargement of the costochondral junctions. There were no fractures of any of the bones.

Two of the rats on diet *D* unfortunately died early in the experiment of acute enteritis. The remaining two (G. 31 and G. 97) were similar to those which showed the least abnormality on diets *B* and *C*.

Slight enlargement of the costochondral junction of the ribs was noticed in rat G. 32 on diet *E*, while the same enlargement, and fractures of several of the ribs were seen in rat G. 66. The bony portion of the ribs and the shaft of the long bones were quite brittle in these two cases. But the remaining two rats, with the exception of slight brittleness of the bones, showed no gross abnormality.

Two of the rats on diet *F* (G. 33 and G. 67) from the same litters as those on diet *E* which showed gross evidence of pathology of the bones, had moderate enlargement of the costochondral junctions and numerous fractures of the ribs, but rats G. 53 and G. 99 showed no gross evidence of disease of the bones except slightly increased fragility. In all the rats on diets *E* and *F* the teeth were moderately opaque, but the opacity was greatest in those rats which showed macroscopical lesions of the bones. It should be noted here that the

Table VI.

No. of rat	Sex	No. of litter	Diet	Age (in days) Initial	Age (in days) Final	Days on diet	Average amount of food eaten daily g.	Weight (g.) Initial	Weight (g.) Final	Gain in g.	In bones % H_2O	In bones % Ca wet	In bones % Ca dry	Histological diagnosis
G. 27	♀♀♀♀♀	XVI	*AA*	21	88	67	16·0	20	155	135	39·39	13·80	22·86	Normal
G. 47	♀♀♀♀♀	XVII	*AA*	21	84	63	15·5	24	168	144	35·99	14·18	22·16	,,
G. 61	♀♀♀♀♀	XVIII	*AA*	22	85	63	13·9	26	133	107	37·70	13·23	21·24	,,
G. 93	♀♀♀♀♀	XIX	*AA*	22	84	62	16·7	23	154	131	36·96	13·48	22·78	,,
										Averages	37·51	13·67	22·24	
G. 28	♀♀♀♀♀	XVI	*A*	21	88	67	14·1	19	136	117	36·87	14·20	22·48	
G. 48	♀♀♀♀♀	XVII	*A*	21	84	63	15·7	26	158	132	36·93	13·98	22·17	,,
G. 62	♀♀♀♀♀	XVIII	*A*	22	85	63	15·5	27	171	144	39·02	13·73	22·52	,,
G. 94	♀♀♀♀♀	XIX	*A*	22	84	62	15·9	22	134	112	39·59	13·82	22·87	,,
										Averages	38·10	13·93	22·51	
G. 29	♀♀♀♀♀	XVI	*B*	21	88	67	14·6	20	128	108	40·33	12·37	20·73	Nearly normal
G. 49	♀♀♀♀♀	XVII	*B*	21	84	63	12·5	24	121	97	45·69	10·62	19·52	Very slight rickets (healing)
G. 63	♀♀♀♀♀	XVIII	*B*	22	85	63	11·0	31	101	70	40·95	10·43	17·67	Slight osteomalacia
G. 95	♀♀♀♀♀	XIX	*B*	22	84	62	15·5	22	123	101	38·70	12·18	19·87	Nearly normal
										Averages	41·41	11·40	19·44	
G. 30	♀♀♀♀♀	XVI	*C*	21	88	67	14·0	18	108	90	40·11	11·82	19·75	Nearly normal
G. 50	♀♀♀♀♀	XVII	*C*	21	84	63	13·1	23	127	104	43·60	10·70	19·13	Slight rickets
G. 64	♀♀♀♀♀	XVIII	*C*	22	85	63	13·1	29	112	83	41·90	10·17	17·49	Moderate osteomalacia
G. 96	♀♀♀♀♀	XIX	*C*	22	84	62	14·5	24	109	85	41·91	11·89	20·47	Nearly normal
										Averages	41·88	11·14	19·21	
G. 31	♀♀♀♀♀	XVI	*D*	21	88	67	10·4	20	77	57	44·01	9·16	16·36	Nearly normal
G. 51	♀♀♀♀♀	XVII	*D*	21	32	11	—	23	21	−2	—	—	—	Died. Enteritis. Osteoporosis severe
G. 65	♀♀♀♀♀	XVIII	*D*	22	62	40	—	25	57	32	—	—	—	Died. Enteritis. Slight rickets, moderate osteoporosis
G. 97	♀♀	XIX	*D*	22	84	62	16·0	25	137	112	40·55	11·98	20·17	Nearly normal
										Averages	42·28	10·57	18·26	
G. 32	♀♀♀♀♀	XVI	*E*	21	88	67	16·3	20	110	90	40·82	12·0	20·28	Slight osteomalacia
G. 52	♀♀♀♀♀	XVII	*E*	21	84	63	12·9	24	97	73	41·82	10·95	18·82	Moderate osteoporosis
G. 66	♀♀♀♀♀	XVIII	*E*	22	85	63	9·9	23	78	55	47·16	8·52	16·12	Very slight rickets and osteoporosis
G. 98	♀♀♀♀♀	XIX	*E*	22	84	62	14·1	26	116	90	26·99	12·27	19·45	Nearly normal
										Averages	41·68	10·93	18·66	
G. 33	♀♀♀♀♀	XVI	*F*	21	88	67	14·7	19	96	77	42·95	9·82	17·21	Slight osteomalacia and osteoporosis
G. 53	♀♀♀♀♀	XVII	*F*	21	84	63	14·6	25	117	92	44·75	10·69	19·26	Slight osteoporosis.
G. 67	♀♀♀♀♀	XVIII	*F*	22	85	63	12·3	28	96	68	42·75	9·53	16·64	Slight rickets and osteoporosis
G. 99	♀♀♀♀♀	XIX	*F*	22	84	62	17·1	27	124	97	36·84	12·14	19·68	Nearly normal
										Averages	41·82	10·61	18·19	

gross lesions above described which manifested themselves in the rats on some of the deficient diets occurred mainly in those from the same two litters XVII and XVIII.

Histology.

The bones of the control rats on diets *AA* and *A* were normal in every respect and no differences were noted between the bones of rats that received butter and those that received cod-liver oil in their food.

On diet *B*, the bones of rats G. 29 and G. 95 from litters XVI and XIX respectively, were practically normal. The trabeculae of the primary spongiosa were not quite as large or as numerous and were not arranged as orderly as those of the perfectly normal bones, but the cartilage and cortex were normal. However, rat G. 49 from litter XVII had very slight rickets and rat G. 63 from litter XVIII slight osteomalacia.

The rats which had diet *C* were similar to those on diet *B* from the corresponding litters.

Of the two rats on diet *D* which died early, one (G. 51) showed severe osteoporosis, and the other (G. 65) rickets and moderate osteoporosis. Rats G. 31 and G. 97 were practically normal, the histological picture being similar to that described for the bones of rats on diet *B* from the same litters.

In the bones of the rats on diets *E* and *F* osteoporosis was a more frequent complicating feature. All the animals on these two diets showed it in some degree (see Pl. V, fig. 3). One case of very slight rickets (G. 66) occurred among the rats on diet *E*, and one of slight rickets (G. 67) among those on diet *F*.

Chemical analysis.

As before, femur, tibia and fibula of both hind limbs were analysed for calcium content. Table VI shows that the bones of the rats on the control diets *AA* and *A* contained the normal amount of water and of calcium for rats of that age. No difference was found between the rats on the diet containing butter and those on the one containing cod-liver oil. In fact, on the average, the bones of rats on the butter diet calcified slightly better than those of rats on the cod-liver oil diet. The average calcium content of these control animals was slightly higher than that of the control rats on diet *A* in the experiments of Part I. Two facts probably explain this difference, (*a*) the greater store of the fat-soluble organic factor in the rats of the last experiments as compared with the first at the time they were placed on the special diets and (*b*) the fact that the butter used in the experiments of Part I was winter butter. Both undoubtedly played a part.

Contrary to what occurred in the experiments reported in Part I, no definite gradation was found in the percentage of calcium in the bones of rats on the various deficient diets. The bones of rats on diets *B* and *C* had practically the same percentage of calcium and of water, while those of the rats on diets *D*, *E* and *F* differed but little in their percentage of calcium and of

water. However, by dividing the seven diets into three groups, some indication of grading can be seen, the average percentage of water in the bones increasing, and that of calcium decreasing slightly as the content of the diet in fat-soluble organic factor diminished. As in the experiments of Part I, the greatest difference was between the normal and the first group of diets deficient in organic factor.

Table VII.

Diets	Average % Ca per wet weight of bones	Average % Ca per dry weight of bones	Deficiency of Ca as % of normal		Average % H_2O in bones	Excess of H_2O as % of normal
			wet bones	dry bones		
AA and *A*	13·79	22·35	—	—	37·80	—
B and *C*	11·27	19·32	18·28	13·56	41·64	10·15
D, *E* and *F*	10·70	18·37	22·41	17·81	41·93	10·92

Summary of Part III.

In order to determine whether a deficiency in the store of the fat-soluble organic factor in the body of the rats prior to the experimental period played a part in the results obtained in the experiments reported in Part I, the same experiments were repeated with rats whose parents had received cod-liver oil, before and during pregnancy, and the entire period of lactation.

The young grew better during the period of lactation, and later felt the effect of the deficient diets much less than the rats of Part I experiments, whose parents did not receive the special breeding paste containing cod-liver oil.

The average percentage of calcium in the bones of the rats on all the diets was higher and the percentage of water lower than on the corresponding diets in the experiments of Part I. There was very little difference between the percentages of calcium and of water in the bones of the rats on the various deficient diets, the gradation in those respects, so marked in Part I experiments, being very slight and apparent only when the diets were divided into three main groups *AA* and *A*; *B* and *C*; *D*, *E* and *F*. It would seem that the store of organic factor which these rats possessed at the time they were put on the special diets was able to make up in great part for its deficiency in those diets.

Despite the increased store of the fat-soluble organic factor which these rats presumably had at the time they were put on the special diets, one or two rats on all the deficient diets developed either slight osteomalacia or slight rickets. Those rats that developed a pathological condition of the bones were all from the same two litters XVII and XVIII.

Part IV.

Finally an investigation was made of the effect of increasing the calcium content of *F* diet, using rats bred from parents whose food contained an abundance of fat-soluble organic factor.

For this a litter of nine rats was employed bred from a mother who, in addition to the ordinary diet of fresh milk, white bread, oats, bran and green cabbage leaf, received daily for many months prior to and during the entire period of pregnancy and lactation, 10 g. of a paste containing fresh summer butter.

This will be referred to as "butter breeding paste" and was constituted as follows:

Butter breeding paste:

"Casein" (commercial)	20 g.	Calcium lactate	0·3 g.
Starch (wheat)	50 g.	Acid potassium phosphate	0·6 g.
Butter	15 g.	Yeast	6·0 g.
Sodium chloride	0·3 g.	Water	67 cc.
Tricalcium phosphate	0·8 g.		

The rats were only three weeks old, averaged 31 g. in weight, and were in excellent condition when they were put on the special diet.

By the addition of calcium in the form of calcium carbonate to diet *F*, diets *F* 1 and *F* 2 were made, the former containing double and the latter four times the quantity of calcium in the original diet *F*. These are identical with diets *F* 1 and *F* 2 used in Part II. Also, by removing some of the calcium lactate from salt mixture No. 185 and replacing this by powdered agar the calcium content of diet *F* was reduced by about one-fourth. This will be referred to as diet *F* 3. The purpose of the latter was to determine whether the calcium content of diet *F* is very near the lower limit.

	Per 100 g. wet diet		Proportion of Ca : P	
Diet	Amount of Ca g.	Amount of P g.	Ca	P
F 3	0·193	0·538	0·375	1·0
F	0·258	0·538	0·479	1·0
F 1	0·538	0·538	1·0	1·0
F 2	1·076	0·538	2·0	1·0

The rats were kept on these diets for eight weeks. All gained weight about equally, those on the diet containing the least amount of calcium (*F* 3) reaching a final weight as high as those of the corresponding sex on the remaining three diets. None reached the normal weight for rats of that age, but all grew better than those on diet *F* in Part I (whose parents were on the ordinary normal diet) (see Table I) and as well as those on diet *F* in Part III (whose parents were on the ordinary normal diet + cod-liver oil breeding paste (see Table VI)). At autopsy, no gross abnormalities of the bones were noted except slightly increased brittleness. In that respect the bones of rats on diet *F* 3 were slightly worse than the others, but no spontaneous fractures occurred in any of the rats. Histologically, no appreciable differences were found between the rats on diets *F*, *F* 1 and *F* 2, their bones being practically normal and showing only a very slight degree of osteoporosis. The bones of

Table VIII.

No. of rat	Sex	No. of litter	Diet	Age (in days) Initial	Age (in days) Final	Days on diet	Average amount of food eaten daily g.	Weight (g.) Initial	Weight (g.) Final	Gain in g.	In bones % H_2O	In bones % Ca wet	In bones % Ca dry	Histological diagnosis
G. 398	♂	xx	*F* 3	22	77	55	12·5	33	132	99	38·77	11·92	19·47	Slight osteoporosis
G. 399	♀	xx	*F* 3	22	77	55	12·5	31	107	76	39·55	12·15	20·12	Nearly normal
G. 400	♂	xx	*F* 3	22	77	55	13·1	31	97	66	40·05	10·23	17·09	Moderate osteoporosis
										Averages	39·45	11·43	18·89	
G. 401	♂	xx	*F*	22	77	55	14·5	33	116	83	39·88	11·85	19·72	Nearly normal
G. 402	♀	xx	*F*	22	77	55	14·5	30	117	87	38·75	13·29	21·21	,, ,,
										Averages	39·31	12·57	20·46	
G. 403	♂	xx	*F* 1	22	77	55	13·2	31	139	108	40·65	12·09	20·80	Nearly normal
G. 404	♀	xx	*F* 1	22	77	55	13·2	30	92	62	38·50	12·12	19·71	,, ,,
										Averages	39·57	12·10	20·25	
G. 405	♂	xx	*F* 2	22	77	55	11·4	31	108	77	39·80	12·32	20·49	Nearly normal
G. 406	♀	xx	*F* 2	22	77	55	11·4	28	83	55	36·87	12·40	19·65	Slight osteoporosis
										Averages	38·33	12·36	20·07	

the rats on diet *F* 3 were more osteoporotic than the others. But no histological signs of rickets or of osteomalacia were present in the bones of any of the rats. The percentage of calcium and of water was practically the same in the bones of all the rats (see Table VIII), but the calcium was slightly less and the water slightly more than normal for rats of that age. Those on diet *F* 3 contained on the average a little less calcium than the others, which corresponds with the histological results.

Table IX.

	In bones		
Diet	Average % H_2O	Average % Ca wet	Average % Ca dry
F 3	39·45	11·43	18·89
F	39·31	12·57	20·46
F 1	39·57	12·10	20·25
F 2	38·33	12·36	20·07

Summary of Part IV.

When rats containing a large store of fat-soluble organic factor are used, increasing (double and four times) the calcium content of diet *F*, *i.e.* 0·258 g. per 100 g. wet diet, does not increase the percentage of calcium deposited in the bones. But a decrease of the calcium content of diet *F* by about 25 % (diet *F* 3) shows a corresponding slight decrease in the percentage of calcium deposited in the bones.

The percentage of calcium in diet *F* (used in Part I and Part II experiments) is adequate, but it is rather close to the lower limit of requirement.

Final Conclusions.

1. When rats are used which have only a *small* "store" of fat-soluble organic factor (rats bred from parents on the ordinary normal diet), grading the content of that factor in their diet results in a corresponding gradation of the percentage of calcium in their bones. Histologically, some of the rats on the diets very deficient in fat-soluble organic factor show slight to severe osteomalacia or rickets (Part I).

2. When rats are used which have a *large* "store" of fat-soluble organic factor (bred from parents who, in addition to the ordinary normal diet, received the cod-liver oil breeding paste), grading the content of that factor in their diet results in only the slightest gradation of the percentage of calcium in the bones. Even in some of these well-bred rats, on the diets deficient in organic factor, very slight osteomalacia or rickets may develop (Part III).

3. In the absence of exposure of the animals to direct sunlight or artificial substitutes for it, the fat-soluble organic factor in the diet, by itself, plays an important rôle in the mechanism for the deposition of calcium in the bones of rats. The reduction below a certain level of the quantity of fat-soluble organic factor in an otherwise complete diet results in a diminished calcium deposit in the bones.

Fig. 1.

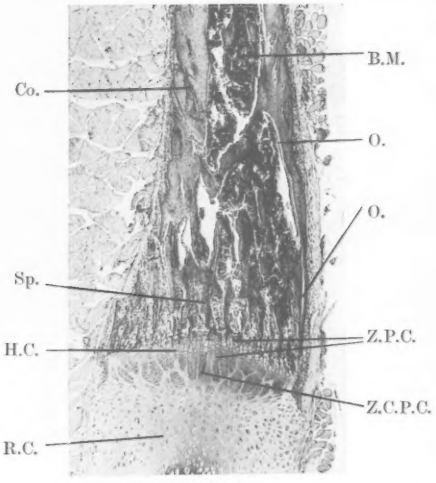

Fig. 2.

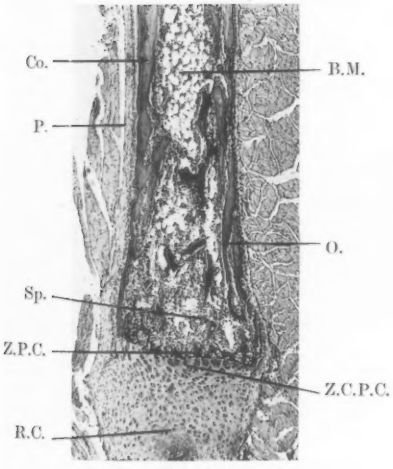

Fig. 3.

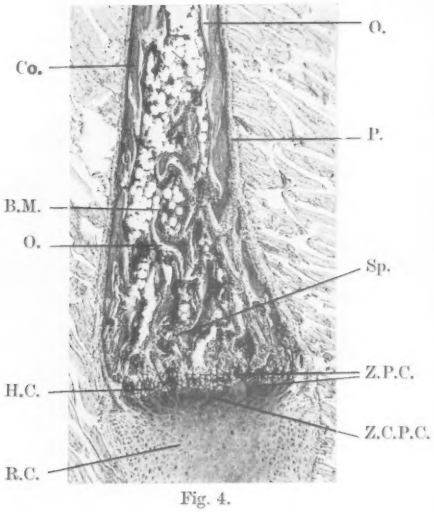

Fig. 4.

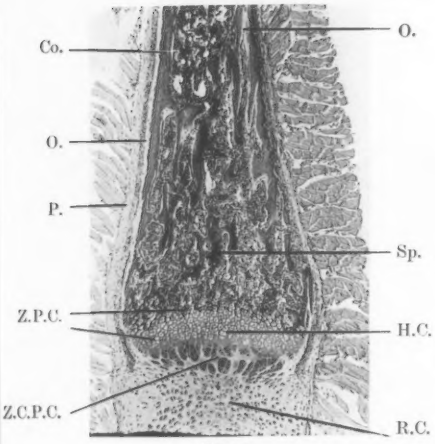

Fig. 5.

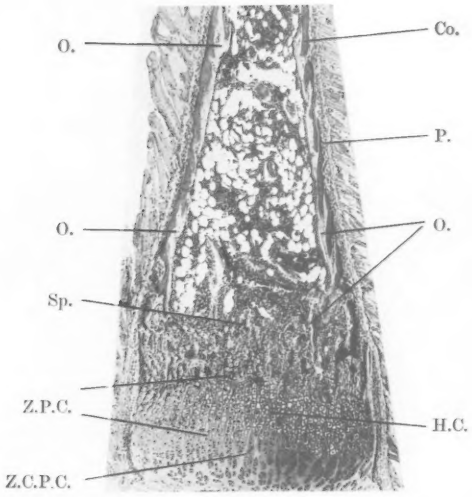

Fig. 6.

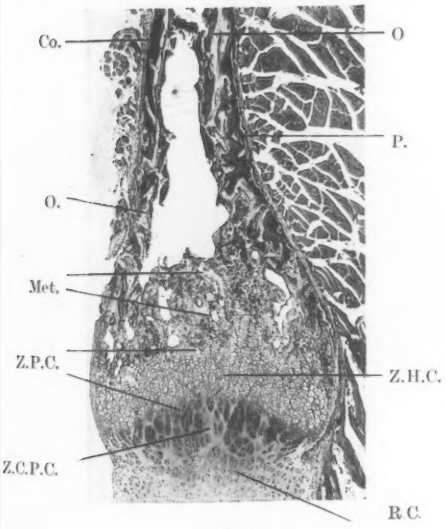

Fig. 7.

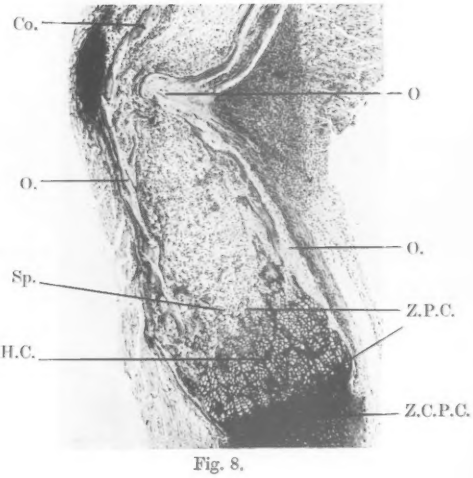

Fig. 8.

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Goldblatt, p. 325. In the legend of Fig. 8. *for* "Moderate rickets" *read* "Severe rickets and slight osteoporosis."

4. A normal diet containing 10 % (per wet weight) of fresh summer butter, proved as good as one containing an optimal quantity (3·6 %) of cod-liver oil, in causing normal growth and the normal development and calcification of the bones of rats.

5. The calcium content of the diets, although rather close to the lower limit, is adequate, but probably not optimal and certainly not excessive (Part II and Part IV).

Finally, it is a sincere pleasure to acknowledge my thanks to Prof. C. J. Martin and Prof. V. Korenchevsky for their kind interest and advice during the conduct of this investigation; to Miss K. M. Soames for very able technical assistance, and to Misses S. Rutherford, M. Pickersgill, and D. Russell for help in feeding and taking care of the animals at various times during the experiments.

EXPLANATION OF FIGURES.

The figures in Plates V and VI are from the rats of these experiments, and were chosen to illustrate the diagnoses given in the tables.

Abbreviations:

Co.	Calcified cortical bone.	*Z.P.C.*	Zone of provisional calcification.
Sp.	Spongiosa.	*H.C.*	Hypertrophic cartilage cells.
O.	Osteoid.	*B.M.*	Bone marrow.
P.	Periosteum.	*Met.*	Metaphysis.
Z.C.P.C.	Zone of proliferous cartilage.		

Fig 1. Rat R. 350 (Part I). Costochondral junction of rib. Diet *A*. Normal Rat. Age 83 days. Weight 177 g. Normal calcification of cortical bone, trabeculae and zone of provisional calcification. Trabeculae of primary spongiosa numerous and regularly arranged. Very little osteoid. Normal rib. Haem.-eosin. Magn. × 32.

Fig. 2. Normal rat. G. 27 (Part III). Rib. Description same as Fig. 1. Haem.-eosin. Magn. × 32.

Fig. 3. Rat G. 52 (Part III, Table VI). Rib. Thin cortex. Very few trabeculae in primary spongiosa. Intense calcification of *Z.P.C.* Very little osteoid. Moderate osteoporosis. Haem.-eosin. Magn. × 32.

Fig. 4. Rat G. 63 (Part III, Table VI). Rib. Note increase of osteoid. Zone of provisional calcification normally calcified. Slight osteomalacia. Haem. and eosin. Magn. × 32.

Fig. 5. Rat G. 49 (Part III, Table VI). Note increase in size of zone of provisional calcification, the cells nearest the spongiosa showing fair calcification of their matrix. Increase of cortical osteoid and that surrounding the trabeculae of the spongiosa. Very slight (healing?) rickets. Haem. and eosin. Magn. × 32.

Fig. 6 Rat G. 69 (Part I, Table I). Rib. Greater width, and imperfect calcification of zone of provisional calcification than in Fig. 5, rat G. 49. Also slightly more osteoid than in the previous figure. But cortex is relatively thinner and trabeculae fewer. Slight rickets and osteoporosis. Haem. and eosin. Magn. × 32.

Fig 7. Rat R. 389 (Part I, Table I). Rib. Note even greater width of zone of provisional calcification than in Fig. 6. Very imperfect calcification and some disorganisation of it by the ingrowth of blood vessels from the marrow. Relatively large amount of osteoid. Moderate rickets. Haem. and eosin. Magn. × 32.

Fig. 8. Rat R. 413 (Part I, Table I). Rib. Note the very great width and imperfect calcification and disorganisation of the zone of provisional calcification. Relatively very large amount of cortical osteoid. Infraction of cortical bone, with deformity. Moderate rickets. Haem. and eosin. Magn. × 32.

BIBLIOGRAPHY.

Aron, H. and Sebauer (1910). *Abderhalden's Handb. der biochem. Arbeitsmethoden*, **1**, 413.
Coward, K. H., Lush, J. L. and Palmer, M. G. (1923). *Lancet*, i, 124.
Hess, A. F., McCann, G. F. and Pappenheimer, A. M. (1921). *Proc. Soc. Exp. Biol.* **18**, 266. *J. Biol. Chem.* **47**, 395.
Hopkins, F. G. (1920). *Biochem. J.* **14**, 725.
Korenchevsky, V. (1921). *Brit. Med. J.* ii, 547.
—— (1922). *Med. Res. Council, Special Report Series*, No. 71, London.
Mackay, H. M. M. (1921). *Biochem. J.* **15**, 19.
McCollum, E. V. (1917). *J. Amer. Med. Ass.* **68**, 1379.
McCollum, E. V., Simmonds, N., Becker, J. E. and Shipley, P. G. (1922). *J. Biol. Chem.* **53**, 19.
McCollum, E. V., Simmonds, N., Shipley, P. G. and Park, E. A. (1922). *Bull. Johns Hopkins Hosp.* **33**, 296.
Mellanby, E. (1918). *J. Physiol.* **52**. *Proc.* p. xi.
—— (1919). *Lancet*, i, 407.
—— (1920). *Lancet*, i, 856.
—— (1921). *Experimental Rickets, Med. Res. Council Special Report Series*, No. 61, London.
Osborne, T. B. and Mendel, L. B. (1915). *J. Biol. Chem.* **20**, 351.
Osborne, T. B. and Mendel, L. (1922). *Proc. Soc. Exp. Biol. Med.* **19**, 87.
Shipley, P. G., McCollum, E. V. and Simmonds, N. (1921). *J. Biol. Chem.* **49**, 399.
Shipley, P. G., Park, E. A., McCollum, E. V. and Simmonds, N. (1921). *Amer. J. Hyg.* **1**, 512.
Tozer, E. (1921). *J. Path. Bact.* **24**, 306.
Zilva, S. S. and Miura, M. (1921). *Biochem. J.* **15**, 654.
Zilva, S. S., Golding, T., Drummond, J. and Coward, K. H. (1921). *Biochem. J.* **15**, 427.
Zucker, Barnett and Johnson (1923). *Proc. Soc. Exp. Biol. Med.* **20**, 20.

XXXVI. THE FERMENTATION OF GLUCOSE AND FRUCTOSE BY DRIED YEAST IN THE SIMULTANEOUS PRESENCE OF PHOSPHATE AND SULPHITE.

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Neuberg has conclusively demonstrated in a long series of brilliant researches that when sugar is fermented by yeast, or by yeast preparations such as dried yeast or maceration extract, acetaldehyde is formed as an intermediate product and is then converted by reduction into alcohol. When the acetaldehyde is "fixed" by the addition of a sulphite, another intermediate product is reduced with the formation of glycerol,

$$C_6H_{12}O_6 = CO_2 + C_2H_4O + C_3H_8O_3,$$

and the fermentation is thus enabled to continue.

Now it has been shown by Harden and Young [1910] that in the normal fermentation of sugar by yeast preparations hexosediphosphate is formed according to the equation:

$$2C_6H_{12}O_6 + 2R_2HPO_4 = 2CO_2 + 2C_2H_6O + C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O.$$

This is regarded by these authors as also representing the course of fermentation in the yeast cell, whereas Neuberg regards it as a pathological phenomenon which does not occur normally in the yeast cell, but only when the cell has been injured in some way, by drying, grinding, action of toluene, etc. Under these circumstances it was thought to be of interest to examine the effect of the presence of phosphate on the production of aldehyde by yeast preparations in the presence of sulphite, in order to ascertain whether either of these two changes in any way interfered with the progress of the other.

The results show that the addition of phosphate in no way interferes with the production of aldehyde. On the contrary the percentage of aldehyde, as calculated on the sugar actually fermented is usually slightly greater in the presence than in the absence of added phosphate. The fermentation of potassium hexosephosphate itself also yields aldehyde in presence of sulphite.

Further, the presence of sulphite does not prevent esterification of the phosphoric acid (see Exps. 5 and 6) so that the two processes in no way interfere. This is consistent with the view of Harden and Young that the phosphate is concerned in the early stages of the decomposition of the sugar molecule.

Experimental.

In order to obtain a series of comparable results dried yeast was employed in presence of toluene. Three different samples of dried yeast (Nos. 1, 2, 3) were used at different times. They were all prepared by pressing out fresh brewer's yeast at about $\frac{1}{2}$ ton per sq. inch and then drying at 37° for 48 hours.

The experiments were carried out by placing 5 g. of the dried yeast + 25 cc. H_2O + 0·2 cc. toluene + 1 (or sometimes 2) g. of the sugar in a flask and incubating at 30° in a thermostat, the CO_2 evolved being measured. After an interval, usually of 1 hour, additions were made in the form of 0·6M K_2HPO_4, a solution of 20 g. $Na_2SO_3 . 7H_2O$ per 100 cc. and if necessary water, all previously saturated at 30° with CO_2. No estimations of p_H were made.

After the requisite period of incubation, during which any CO_2 evolved was measured, the contents of the flask were cooled in ice and made up to 100 cc. One portion of 50 cc. was filtered and 20 cc. taken for the estimation of inorganic phosphate by means of magnesium citrate solution and ammonia. The results are expressed as mgm. of K_2HPO_4. In the remainder the acetaldehyde was estimated by the method described by Neuberg [1918]. Excess of barium chloride solution was added, the mixture allowed to stand in the cold, filtered and a portion of the filtrate distilled with $CaCO_3$ and the aldehyde estimated in the distillate.

Another portion (10 cc.) of the filtrate was taken for the estimation of reducing sugar by Bertrand's method, after precipitating the excess of barium by sodium sulphate.

The amount of sugar thus obtained does not however represent the total sugar which has escaped fermentation to CO_2 and either alcohol or aldehyde. In the first place any hexosediphosphate present at the close of fermentation would be almost completely precipitated by the barium chloride and in the second place any hexosemonophosphoric acid [Robison, 1922] would still be present, but would have a reducing power considerably less than that of free glucose or fructose. Further information can be obtained from a consideration of the amount of inorganic phosphate in the liquid, the difference between this and the total of the amounts added and originally present in the yeast representing phosphate combined either as hexosediphosphate or hexosemonophosphate.

In order to obtain an approximate idea of the amount of sugar fermented it has been assumed that the combined sugar is all present as hexosediphosphate.

In the first place it was found, in agreement with Neuberg's results [1919], that the addition of sulphite must be made after a vigorous sugar fermentation had commenced. When the sulphite was added along with the sugar at the commencement of the experiment, fermentation did not ensue, and even when the addition was made at the end of 30 minutes very little action occurred. When an hour was allowed to elapse before the addition was made,

satisfactory results were obtained. This is clearly shown in the following experiment.

Exp. 1. 5 g. of dried yeast (No. 2) + 1 g. glucose + 25 cc. H_2O + 0·2 cc. toluene, incubated at 30°.

To four series of flasks the following additions were made after intervals of 5, 30 and 60 minutes from the commencement of incubation and the mixtures were incubated for a further period of six hours.

No.	0·6*M* K_2HPO_4 cc.	20 % $Na_2SO_3.7H_2O$ cc.	H_2O cc.
1	5	2·5	2·5
2	0	2·5	7·5
3	5	5	0
4	0	5	5

Results.

No.	Time of addition mins.	Glucose mgm.		Free phosphate as K_2HPO_4 mgm.		Acetaldehyde mgm.
		Initial	Final	Initial	Final	
1	5	1000	1014	900	826	0
	30	916	923	880	791	5·1
	60	864	422	820	668	10·9
2	5	1000	1005	380	366	0
	30	916	970	360	383	4·5
	60	864	0	300	337	32
3	5	1000	1005	900	820	0
	30	916	920	880	781	8·3
	60	864	630	820	708	14·7
4	5	1000	1005	380	374	0
	30	916	978	360	381	7·7
	60	864	702	300	358	12·8

The initial figures are calculated from the amount of CO_2 evolved during the preliminary incubation.

In all subsequent experiments therefore the sulphite was added at least one hour after the fermentation had commenced.

It was also found that both phosphate and sulphite tended to stop the fermentation so that it was necessary to ascertain the best amounts to add. It was not found possible to use more than 5 cc. of 20 % sulphite (= 0·5 g. Na_2SO_3) + 10 cc. of 0·6*M* K_2HPO_4 (= 1·045 g. K_2HPO_4) with 5 g. dried yeast in a total volume of about 40 cc., and even this was excessive and resulted in a very small amount of fermentation. This only amounts to a concentration of sulphite of 1·25 g. Na_2SO_3 per 100 cc., whereas Neuberg (with living yeast) used 12·6 g. per 100 cc. This amount of sulphite is theoretically capable of combining with 174 milligrams of aldehyde corresponding with 0·73 g. of sugar fermented according to Neuberg's equation. In many experiments only 2·5 cc. of 20 % sulphite and 5 cc. of 0·6*M* K_2HPO_4 were used and in some only 1·25 cc. of sulphite.

The following is a typical experiment in which 5 cc. of sodium sulphite and 10 cc. of K_2HPO_4 were used.

Exp. 2. 5 g. dried yeast (No. 1) + 1 g. glucose + 25 cc. H_2O + 0·2 cc. toluene.

After 75 mins. incubation the following additions were made to two series of flasks.

No.	0·6*M* K_2HPO_4 cc.	20 % $Na_2SO_3.7H_2O$ cc.	H_2O cc.
1	10	5	0
2	10	0	5
3	0	5	10
4	0	0	15

After the addition the flasks of Series 1 were incubated for three hours and those of Series 2 for six hours.

Results.

No.	Incubation period	Residual glucose mgm.	Phosphate as K_2HPO_4 mgm.			Acetaldehyde mgm.
			Total	Free	Combined	
1	3	476	1405	1105	300	10·1
	6	450	1405	1271	134	16·8
2	3	50	1405	1193	212	0
	6	0	1405	1304	101	0
3	3	512	360	310	50	9·1
	6	492	360	360	0	11·2
4	3	100	360	202	158	0
	6	0	360	228	132	0

The sugar present (free and combined) at the moment when the additions were made was 0·65 g. and the phosphate content of the dried yeast was 360 mgm. (K_2HPO_4).

In this case the amount of fermentation was greatly reduced by the addition of the sulphite, compare 1 and 2, 3 and 4. The amount of phosphate is also somewhat excessive.

Other experiments in which less sulphite was employed are the following:

Exp. 3. 5 g. dried yeast (No. 2) + 1 g. glucose + 25 cc. H_2O + 0·2 cc. toluene at 30°.

After one hour's incubation, the following additions were made and incubation continued for six hours.

No.	0·6*M* K_2HPO_4 cc.	20 % $Na_2SO_3.7H_2O$ cc.	H_2O cc
1	5	2·5	2·5
2	2·5	2·5	5
3	0	2·5	7·5
4	5	1·25	3·75
5	0	1·25	8·75
6	0	0	10

After one hour the total remaining glucose (free and combined) was 747 mgm. The analyses gave the following results:

No.	Residual glucose mgm.	Phosphate as K_2HPO_4 mgm.			Acetaldehyde mgm.
		Total	Free	Combined	
1	438	902	669	233	10·9
2	180	641	389	252	13·5
3	0	380	344	36	31·7
4	0	902	770	132	35·0
5	0	380	340	40	28·2
6	0	380	210	170	0

Here in presence of 5 cc. phosphate, 2·5 cc. of Na_2SO_3 solution has greatly diminished the fermentation, whereas in absence of the phosphate the sugar has been practically all fermented away as has also been the case in the presence of 1·25 cc. of Na_2SO_3 even in the presence of 5 cc. of phosphate.

On the assumption that the whole of the combined phosphorus is present as hexosediphosphate, the percentages work out as follows:

No.	Sugar used	Sugar as phosphoric ester	Sugar fermented	Acetaldehyde	Acetaldehyde % of sugar fermented
1	309	121	188	10·9	5·8
2	567	130	437	13·5	3·1
3	747	19	728	31·7	4·3
4	747	68	679	35·0	5·2
5	747	21	726	28·2	3·9

It will be observed that both in presence of 2·5 and 1·25 cc. of sulphite the higher percentage is produced in presence of added phosphate. It must be remembered at the same time that the sulphite in 4 and 5 was only capable of combining with about one-third of the aldehyde theoretically obtainable from the sugar fermented.

Exp. 4. 5 g. dried yeast (No. 2) and 1·933 g. fructose (2 g. of 96·6 %) + 25 cc. H_2O + ·2 cc. toluene.

After one hour's incubation, the following additions were made and incubation continued for six hours.

No.	0·6*M* K_2HPO_4 cc.	20 % $Na_2SO_3.7H_2O$ cc.	H_2O cc.
1	5	2·5	2·5
2	2·5	2·5	5
3	0	2·5	7·5
4	5	1·25	3·75
5	0	1·25	8·75

After one hour the total remaining fructose (free and combined) was 1680 mgm.

Results.

No.	Residual fructose mgm.	Phosphate as K_2HPO_4 mgm.			Acetaldehyde mgm.
		Total	Free	Combined	
1	293	902	209	693	56·3
2	173	641	59	582	42·7
3	348	380	0	380	46·7
4	108	902	484	418	47·4
5	422	380	0	380	33·9

Treating these figures as those in Exp. 3, the following percentages are obtained.

No.	Fructose used	Fructose as phosphoric ester	Fructose fermented	Acetaldehyde	
				mgm.	% of fructose fermented
1	1387	359	1028	56·3	5·5
2	1507	301	1206	42·7	3·5
3	1332	197	1135	46·7	4·1
4	1572	216	1356	47·4	3·5
5	1258	197	1061	33·9	3·2

Exp. 5. 5 g. dried yeast (No. 2) + 1·933 g. fructose (2 g. of 96·6 %) + 25 cc. H_2O + ·2 cc. toluene at 30°.

After one hour's incubation the following additions were made and incubation continued for nine hours.

No.	0·6*M* K_2HPO_4 cc.	20 % $Na_2SO_3.7H_2O$ cc.	H_2O cc.
1	5	2	3
2	5	2·5	2·5
3	0	2·5	7·5
4	5	3	2
5	0	3	7

After one hour the total remaining fructose (free and combined) was 1680 mgm.

Results.

No.	Residual fructose mgm.	Phosphate as K_2HPO_4 mgm.			Acetaldehyde mgm.
		Total	Free	Combined	
1	72	902	675	227	62·7
2	87	902	458	444	74·2
3	231	380	153	227	66·0
4	216	902	311	591	65·3
5	159	380	15	365	51·1

Treating these figures as those in Exp. 3, the following percentages are obtained.

No.	Fructose used	Fructose as phosphoric ester	Fructose fermented	Acetaldehyde	
				mgm.	% of fructose fermented
1	1608	·117	1491	62·7	4·2
2	1593	230	1363	74·2	5·4
3	1449	117	1332	66·0	5·0
4	1464	306	1158	65·3	5·6
5	1521	189	1332	51·1	3·8

In No. 1 the amount of aldehyde is not far from the maximum capable of being combined with the sulphite present.

Experiments were also made to ascertain whether fructose, which reacts more readily than glucose with phosphate, would yield aldehyde more or less readily than glucose (Exp. 6) and whether hexosephosphate was also fermented with production of aldehyde (Exp. 7).

Exp. 6. 5 g. dried yeast (No. 3) + 25 cc. water + 0·2 cc. toluene and in (1) and (2) 2 g. glucose and in (3) and (4) 1·97 fructose.

After one hour's incubation the following additions were made and incubation continued for nine hours.

No.	0·6*M* K_2HPO_4 cc.	20 % $Na_2SO_3.7H_2O$ cc.	H_2O cc.
1	5	2·5	0
2	0	2·5	5
3	5	2·5	0
4	0	2·5	5

Results.

No.	Residual sugar mgm.	Phosphate as K_2HPO_4 mgm. Total	Free	Combined	Acetaldehyde mgm.
1	0	902	773	129	75·8
2	0	380	363	17	50·9
3	0	902	794	108	74·9
4	0	380	371	9	53·8

Treating these figures as before:

No.	Sugar used	Sugar as phosphoric ester	Sugar fermented	Acetaldehyde mgm.	Acetaldehyde % of sugar fermented
1	1681	67	1614	75·8	4·7
2	1699	9	1690	50·9	3·0
3	1636	56	1580	74·9	4·7
4	1629	5	1624	53·8	3·3

Glucose and fructose appeared to act exactly alike. In both cases the percentage production in presence of added phosphate is appreciably greater than in its absence.

Exp. 7. In this experiment, in order to ascertain whether the decomposition product of hexosediphosphate had any special significance for the production of aldehyde, fructose and phosphate were added in such proportion that after the preliminary incubation the whole of the residual sugar should be present in the combined form.

The results obtained (summarised below) were of the same order as when free glucose or fructose was fermented.

No.	Time of incubation hours	Sulphite cc.	Sugar fermented calculated from hexosephosphate decomposed mgm.	Acetaldehyde mgm.	Acetaldehyde % of sugar fermented
1 (*a*)	6	2	106	10·2	9·7
(*b*)	6	2·5	108	10·9	10·5
2 (*a*)	24	2	216	11·5	5·3
(*b*)	24	2·5	222	12·2	5·5

Summary.

When the fixation method of Neuberg is applied to the fermentation by dried yeast of glucose or fructose, acetaldehyde is produced to about the same extent in the presence or absence of added phosphate.

I wish to thank Prof. A. Harden for advice and assistance during the prosecution of this investigation.

References.

Harden and Young (1910). *Proc. Roy. Soc.* B. **82**, 321.
Neuberg (1918). *Biochem. Z.* **89**, 365.
—— (1919). *Ber. deutsch. chem. Ges.* **52** B. 1677.
Robison (1922). *Biochem. J.* **16**, 809.

XXXVII. THE RELATION BETWEEN URIC ACID AND ALLANTOIN EXCRETION IN HYBRIDS OF THE DALMATIAN HOUND.

By the late HERBERT ONSLOW.

From the Biochemical Laboratory, Cambridge.

Report to the Medical Research Committee.

(*Received February 19th, 1923.*)

The research recorded in the present communication[1] was suggested by the remarkable fact, based on an observation made by Benedict [1916], that the Dalmatian breed of dog, *i.e.* the spotted coach dog, differs from other dogs in excreting a much higher percentage of uric acid. The late author thought, therefore, it would be interesting to ascertain how offspring from a cross between the Dalmatian and a normal dog would behave in this respect.

Uric acid and allantoin excretion in mammals.

In the group of mammals there are apparently three products of purine metabolism which may be excreted, *i.e.* uric acid, allantoin and purine bases. Though none of the three, so far as we know, is entirely absent from the urine, the proportion in which they are excreted varies considerably in different species of mammals. For the present purpose we are concerned only with the relative proportions of uric acid and allantoin. From the urine of man and the anthropoid ape, allantoin is almost, though not entirely, absent, whereas uric acid is present to a considerable extent. On the other hand, the urine of the dog contains very appreciable quantities of allantoin but small amounts only of uric acid. This variation in products is correlated with the existence, in certain tissues of the dog, of an enzyme which catalyses (and this may be demonstrated *in vitro*) the oxidation of uric acid to allantoin. This power of destroying uric acid is absent from the tissues of man and the higher apes. The capacity for destroying uric acid has been expressed by what is termed the "uricolytic index," that is the percentage ratio of allantoin nitrogen to the sum of allantoin nitrogen and uric acid nitrogen. The uricolytic index of the dog is about 98, that of man about 2. Of the majority of mammals investigated, the index is near 98, but in some it has been found to be as low

[1] The estimations included in this paper were carried out by Mr John Lowndes, assistant to the late Mr Onslow. Great credit is due to Mr Lowndes for his careful work and attention to the dogs, without which the research would not have been sufficiently complete for publication. (M. W. O.)

as 79 or 80 [Hunter and Givens, 1914; Hunter, Givens and Guion, 1914; Hunter and Ward, 1920]. As mentioned above, Benedict first discovered that the Dalmatian differs from the ordinary dog in regard to its excretion of uric acid, and this observation has been confirmed by Wells [1918]. Benedict observed that, on addition of acid to the urine of such a dog, a heavy precipitate of uric acid crystallised out. He found the high production of uric acid to be exhibited by four dogs of pure breed, but not by a fifth individual of doubtful breed. For the greater part of a year, a Dalmatian weighing about 10 kilos. was kept on a purine-free diet, and the uric acid determined almost daily. The following percentages and indices have been calculated from the data presented in Benedict's paper.

Table I.

Total nitrogen of urine in g.	Percentage of total nitrogen as		Uricolytic index	Remarks
	Uric acid nitrogen	Allantoin nitrogen		
4·8	2·56	—	—	Diet contains 5·9 g. total nitrogen
5·0	2·38	1·14	32	
4·9	2·57	1·31	34	
4·9	2·47	1·24	33	
5·1	2·35	1·00	30	
16·6	0·70	0·22	24	Diet contains 24·07 g. total nitrogen
18·4	0·65	0·58	47	
19·8	0·65	0·52	44	
17·5	0·70	0·58	45	
15·2	0·76	0·55	42	
5·4	2·85	1·35	32	Diet contains 2·03 g. total nitrogen
4·1	3·39	—	—	
3·4	4·09	2·00	33	
3·0	4·70	1·80	28	
2·8	4·96	2·11	30	
2·7	5·18	2·11	29	
5·37	2·40			— —
5·65	2·23	3·74	62	
6·07	2·11			
5·52	2·26			Injection of 100 mgm. caffeine
5·41	2·09	3·56	62	
5·23	2·22			
4·37	2·75	1·14	29	Normal diet
4·54	2·75	1·10	29	
4·82	2·63	1·04	28	
6·48	2·73	3·24	54	Same diet + 100 g. thymus daily = 0·32 g. purine nitrogen
6·86	2·87	3·06	52	
7·30	2·74	2·88	51	
5·75	2·24	1·01	31	Normal diet
5·54	2·38	1·05	31	
5·27	2·60	1·10	30	
5·18	5·60	2·22	28	Same diet + 500 mgm. of uric acid subcutaneously
5·13	5·83	2·77	32	
5·00	6·10	2·66	30	
5·02	2·47	1·31	35	— —

From Table I we see that the uricolytic index of the dog used by Benedict varied from 24–62 according to the diet and other circumstances,

Object of research in the present paper.

As already stated, the object of the research recorded in the present communication was to ascertain how the lack of power of oxidation of uric acid was inherited in a hybrid animal produced by crossing the Dalmatian breed with a normal dog. For this purpose, a Dalmatian bitch (see Plate VII, fig. 1) of pure breed was obtained[1]. She was subsequently mated with a large white terrier dog. Two hybrid offspring were born, a dog (see Plate VII, fig. 2) and a bitch (see Plate VII, fig. 3). Both had the build of a Dalmatian; the dog was entirely without spots, but the bitch was very slightly spotted with small black spots. A metabolism cage was used in the case of three of the dogs, but it was not found possible to put the Dalmatian in the cage.

Method of procedure and estimation.

The urine was collected in a vessel placed under the cage, and at the end of 24 hours the volume and specific gravity were taken. The floor of the cage was then carefully washed with distilled water, and the washings added to the urine. The mixture was acidified[2] with 5 cc. of 20 % sulphuric acid, and the whole made up to 500 cc. (250 cc. or less, in a few cases when the volume of urine was small). This dilution, together with the subsequent dilution when estimating the allantoin, ensures that the urea is not present to an extent greater than 1 %; otherwise it may be precipitated with the allantoin. This was verified by estimations of urea by the hypobromite and urease methods. It was also corroborated by calculating the percentage of urea likely to be present. This is estimated as 1·5 to 3 % as follows: In the dog, the total nitrogen, by volume, is 1 to 2 % of the urine[3], and, of this, the urea nitrogen forms 75 % (*i.e.* 0·75 to 1·5 g. of urea nitrogen per cent., or 1·5 to 3 g. of urea in 100 cc. of the urine). The sulphuric acid was added, because allantoin is destroyed in alkaline solution, and the urine tends to become alkaline on standing [Givens, 1914]. It was not thought advisable to dilute the whole 24 hour specimen to the volume necessary for the allantoin estimation, as this would have caused the uric acid to be present in too great a dilution for accurate determination.

It was noticed, however, on acidifying some samples from the pure Dalmatian that a deposit of uric acid was formed, if allowed to stand, and might, in this way, be lost during the preliminary processes. Practically all these estimations, therefore, were carried out immediately, without acidification.

The uric acid was estimated by the method of Hopkins and Cole [Cole, 1920] and the allantoin by that of Wiechowski [1913]. All estimations were made in duplicate.

[1] From Mrs H. Wilson Bedwell, Heckfield, Hants.

[2] Except in the case of the Dalmatian as mentioned later.

[3] In the case of the terrier recorded later, it was higher, due probably to the long period in the cage, *i.e.* 40 hours.

Estimation of total nitrogen. This was carried out according to Kjeldahl. As a rule, 5 cc. of urine, after the first dilution, were used.

Estimation of uric acid. Of the diluted urine, 300 cc. were taken (with one or two exceptions) and 60 cc. of 0·6 % colloidal ferric hydroxide were added which removes an unknown substance otherwise precipitated later by ammonium chloride. Duplicate 150 cc. portions of the filtrate were used. 2 g. of solid ammonium chloride were added for every 10 cc. of filtrate and stirred until dissolved, and then 3 cc. of strong ammonia. This was allowed to stand, after thoroughly stirring, for as long as 12 hours, when the uric acid was precipitated. The precipitate was filtered off, and washed with 10 % ammonium sulphate in 1 % ammonia solution to remove chloride. It was then washed into 100 cc. of hot water and 20 cc. of 45 % sulphuric acid were added. This was titrated with standard potassium permanganate solution, keeping the mixture at 65°, until the end point is noted by a pink flush which appears all through the liquid.

The amount of uric acid present can be calculated from the permanganate used. The results are expressed in terms of uric acid nitrogen, 1 cc. of $N/20$ permanganate solution being equivalent to 3·70 mgm. of uric acid.

Estimation of allantoin. To 100 cc. of the already diluted urine, 3 cc. of concentrated sulphuric acid and 7·5 cc. of glacial acetic acid were added, and the volume made up to 300 cc. Then excess of solid phosphotungstic acid was added (the necessary total amount having been previously calculated by using test portions of 2 cc. with 50 % phosphotungstic acid) and the whole stirred and allowed to stand about 12 hours. The precipitate was filtered off, and, to the filtrate, which was quite clear, lead oxide was added with constant stirring until the reaction was alkaline. The lead oxide, in the presence of acetic acid, forms both normal and basic lead acetates, and by these the phosphotungstic and sulphuric acids are removed and also certain constituents of the urine. The precipitate was filtered off, and the filtrate should give no further precipitate when tested with lead acetate solution. From 150 cc. of the filtrate the chlorides were then removed as follows. The liquid was made distinctly acid with acetic, and 1 % silver acetate solution was added until there was no further precipitate. The volume was made up to 200 cc. and allowed to stand some hours. It was then filtered, and sulphuretted hydrogen passed through the filtrate until the lead and mercury were completely precipitated. It was filtered again and the sulphuretted hydrogen removed by means of a current of air. Magnesium oxide was added until the filtrate was alkaline to litmus. It was finally filtered, and to 20 cc. of the filtrate the calculated amount of allantoin reagent (5 g. of mercuric acetate and 20 g. of sodium acetate made up to 100 cc.) was added. To determine the necessary amount of allantoin reagent, test portions were treated with the reagent until the latter gave no further precipitate. The filtrate from this, however, should give a flocculent precipitate with a very dilute solution of freshly-prepared allantoin. The precipitate of allantoin was

then filtered off and washed free from urea (the mercury compound of which is decomposed by water), until the washings gave no further precipitate with mercuric nitrate (test for urea). The nitrogen in the allantoin precipitate was estimated by Kjeldahl's method, using 10 % hyposulphite solution (2 cc. of 10 % of hyposulphite for every gram of mercury salt used). All estimations were made in duplicate.

Observations on the parents (Dalmatian and terrier).

It was not possible to put the Dalmatian into the metabolism cage, as she had not been trained and was, moreover, too large when the experiments commenced. Hence, samples of urine were collected at random. She was fed on a mixed and very liberal diet. When the experiments commenced on November 3rd, 1921, she was about 1 year and 8 months old. At the end of the experiments in 1922, her weight was 19 kilos (42 lbs.). The following is a record of the observations made for the Dalmatian and the terrier.

Table II.

Date of experiment	Volume of urine cc.	Sp. gr. of urine	In whole volume of urine: Total nitrogen g.	Uric acid nitrogen g.	Allantoin nitrogen g.	Uricolytic index	% of total nitrogen as: Uric acid nitrogen	Allantoin nitrogen
Experiments with Dalmatian.								
1921								
(1) Nov. 3	180	1·042	—	0·082	0·213	72	—	—
(2) Nov. 5	158	1·028	2·40	0·051	0·257	83	2·11	10·72
(3) Dec. 1 and 7	115	1·037	1·79	0·040	—	—	2·21	—
1922								
(4) Oct. 12	180	1·020	2·33	0·029	0·169	85	1·26	7·26
(5) Oct. 18	320	1·025	5·85	0·090	0·601	87	1·53	10·26
(6) Oct. 19	340	1·018	3·89	0·049	0·645	93	1·25	16·57
(7) Nov. 8	190	1·018	2·33	0·067	0·461	87	2·88	19·78
(8) Nov. 9	60	1·018	0·80	0·026	0·124	83	3·29	15·60
						Average 84	2·08	13·36
Experiment with Terrier.								
1921								
(9) Nov.	110	1·040	3·67	0·010	0·703	99	0·26	19·97

Except in experiment 4, the urine was not acidified. In this experiment a certain amount of uric acid was precipitated on acidification, and this was filtered off, before adding colloidal ferric hydroxide, and estimated separately. There was no preliminary dilution of the urine in experiments 1, 2, 3 and 9. In experiments 4, 5 and 6, it was diluted to 500 cc.; in experiments 7 and 8 to 250 cc. and 150 cc. respectively.

From Table II it will be seen that, for the Dalmatian, the figures are in some respects very different from those calculated in the case of Benedict's dog. Though the results are vitiated in the present paper by the fact that 24 hour samples could not be obtained, it is obvious that a high percentage of uric acid excretion is possible, in fact crystals of uric acid were obtained on adding acid to the urine. The abundant diet is without doubt responsible for the high percentage of allantoin excretion. Umeda [1915] has shown that it may rise from 7 to 16 % on feeding with a diet rich in carbohydrate.

In the case of the Dalmatian, the greater the allantoin values, the higher will be the uricolytic index, whereas in the case of the normal dog, the uric acid value is so small that the allantoin may vary within wide limits without greatly affecting the value of the index. In any case it will be seen that the average figure for uric acid nitrogen expressed as a percentage of the total nitrogen is of the same order as in the case of Benedict's dog. It is much higher than what is found in normal dogs. The values should be compared with those obtained from the terrier with which the Dalmatian bitch was crossed and with those given by the hybrid offspring.

It was only possible to make one observation on the terrier. For this purpose he was kept in the cage for 40 hours. The results are apparently quite normal.

Observations on the offspring.

The two hybrid offspring were trained for the cage, and each experiment was of 24 hours' duration (with the exception of experiment 12 which only lasted 12 hours). They were fed upon the same diet as the Dalmatian. At the beginning of the experiments they were six months old. During the experiments the average weight was 13·6 kilos (30 lbs.) for the dog and 14·5 kilos (32 lbs.) for the bitch. The following is a record of the observations made on the two hybrid offspring.

Table III.

Date of experiment	Volume of urine cc.	Sp. gr. of urine	In whole volume of urine: Total nitrogen g.	Uric acid nitrogen g.	Allantoin nitrogen g.	Uricolytic index	% of total nitrogen as: Uric acid nitrogen	Allantoin nitrogen
			Experiments with Hybrid Dog.					
1922								
(10) July 27–28	210	1·035	4·46	0·011	0·812	99	0·24	18·19
(11) Aug. 9–10	480	1·020	6·43	0·015	1·348	99	0·24	20·94
(12) Sept. 18	210	1·022	2·98	0·008	0·517	98	0·28	17·36
(13) Sept. 21–22	416	1·020	5·57	0·010	0·593	98	0·18	10·64
(14) Sept. 27–28	220	1·050	9·18	0·014	1·378	99	0·16	15·01
					Average	99	0·22	16·43
			Experiments with Hybrid Bitch.					
1922								
(15) July 31–Aug. 1	190	1·020	1·95	0·006	0·406	98	0·32	20·82
(16) Aug. 10–11	430	1·020	6·90	0·027	1·348	98	0·40	19·53
(17) Sept. 19–20	320	1·050	15·05	0·043	1·916	98	0·29	12·73
(18) Sept. 25–26	175	1·020	2·50	0·008	0·470	98	0·34	18·83
(19) Sept. 28–29	260	1·020	4·21	0·010	0·984	99	0·24	23·34
					Average	98	0·32	19·05

The first dilution was in almost all cases to 500 cc. In experiment 12 it was to 250 cc. and in experiment 18 to 300 cc.

It was suggested by Mr S. W. Cole that the sulphuric acid used in acidification, might, as sulphate, interfere with complete precipitation of the ammonium urate. To test this, comparative estimations were made on the same sample, with and without acidification. The results were in complete accordance.

On the evidence of the above results, we may say that in the case of the hybrid Dalmatians the uric acid and allantoin excreted are in relatively the same proportions as in the normal dog. There is possibly a very slight excess of uric acid excreted by the slightly spotted hybrid bitch over the hybrid non-spotted dog. The greater power of destruction of uric acid is therefore a dominant character.

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REFERENCES.

Benedict (1916). *J. Lab. Clin. Med.* **2**, 1.
Cole (1920). Practical Physiological Chemistry.
Givens (1914). *J. Biol. Chem.* **18**, 417.
Hunter and Givens (1914). *J. Biol. Chem.* **18**, 403.
Hunter, Givens and Guion (1914). *J. Biol. Chem.* **18**, 387.
Hunter and Ward (1920). *Trans. Roy. Soc. Canada*, **13** (Sect. 5), 7.
Umeda (1915). *Biochem. J.* **9**, 421.
Wells (1918). *J. Biol. Chem.* **35**, 221.
Wiechowski (1913). Ellinger's Analyse des Harns. **2**, 1076.

Fig. 1. Dalmatian bitch.

Fig. 2. Hybrid dog.

Fig. 3. Hybrid bitch.

XXXVIII. THE HYDROGEN ION CONCENTRATION OF THE BLOOD IN CERTAIN PATHOLOGICAL CONDITIONS, AS DETERMINED BY THE HYDROGEN ELECTRODE AND THE INDIRECT METHODS OF BARCROFT AND HASSELBALCH.

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Hasselbalch [1913] was the first to establish the conditions under which the hydrogen electrode can be used to measure accurately the reaction of the body fluids, and he constructed curves for the p_H of blood under varying CO_2 tensions.

More recently Peters [1914] has improved Hasselbalch's method by using completely reduced blood for the determinations, and Parsons [1917] has shown that by the C_H of blood is meant the C_H of the plasma it contains, *i.e.* the plasma obtained by centrifuging the blood without loss of CO_2—which may be called the "true plasma" [Joffe and Poulton, 1920].

When once the hydrogen electrode apparatus has been set up the determinations do not occupy any great length of time; but as ordinarily arranged the apparatus itself tends to be somewhat complicated. A much simplified arrangement is, however, being designed by one of us (T. R. P.) especially for use with body fluids: it is soon to be placed on the market by the Cambridge and Paul Scientific Instrument Company. In this the manipulations are reduced to a minimum.

Barcroft's method [Barcroft and Peters, 1914] was introduced before the hydrogen electrode method had been finally perfected. It consists in determining at any required CO_2 pressure the percentage saturation with oxygen of the haemoglobin of the blood at a given oxygen pressure, and from this calculating the value of $\log K$ in Hill's equation. Barcroft and Peters [1914] showed for Barcroft's blood that $\log K$ was a linear function of p_H, and Hasselbalch [1916] found that in the case of six normal people the same relationship existed between the two values. Barcroft's method was used by Lewis, Ryffel, Wolf, Cotton and Barcroft [1913] in cases of cardio-renal disease, by Poulton and Ryffel [1913] in cases of uraemia, and by Poulton

[1] This work was carried out during the tenure of a Beit Memorial Research Fellowship.

[1915] in cases of diabetic coma. Hasselbalch [1916, 1917] has pointed out that even if the relationship between p_H and log K holds for normal people, it does not necessarily hold for pathological cases; but unfortunately he did not test the method against the hydrogen electrode, but by calculations from the concentrations of free and combined CO_2 in the blood. In this method of calculating the p_H of blood there is a slight systematic error as pointed out by Parsons [1917].

1. Methods.

Collection of blood, etc. The blood was obtained by venous puncture from patients in Guy's Hospital at about 11 a.m. It was immediately defibrinated, packed in ice inside a vacuum vessel, and sent by passenger train to Cambridge where it was received by T. R. P. at about 2 p.m. The determinations were carried out at once except in cases specially mentioned. The following investigations were made: (1) the p_H of this fully reduced blood at 37° at three or four CO_2 pressures by the method described by Parsons [1917]; (2) the CO_2 content of the same specimen of blood by means of Barcroft's differential apparatus; (3) the log K at the same temperature at varying CO_2 pressures, also with Barcroft's differential apparatus.

The alveolar CO_2 was obtained, by E. P. P. at the time that the blood was taken, by a modification of Hasselbalch and Lindhard's method which is described by Campbell, Hunt and Poulton [1923]. In this series of cases the patient, wearing Bohr's mask and valves, was told to expire deeply towards the end of his normal expiration. Inspiratory samples were not usually taken. In some of the earlier cases the ordinary Haldane-Priestley method was used.

In a few cases E. P. P. determined the CO_2 in the fully or partly oxygenated blood with Van Slyke's apparatus, the CO_2 pressure being measured as described in Joffe and Poulton's paper [1920]. A few determinations of log K were also made by him, according to Barcroft's original method [1914].

2. The relation between log K and p_H in pathological bloods.

This relationship has been carefully tested with the normal blood of Barcroft [1914] and with Parsons' blood, with the addition of lactic acid [Donegan and Parsons, 1919] and by Barcroft and co-workers [1922]. In Fig. 1 the two lines indicate this relationship for the two bloods. The lines are parallel to one another; but they are obviously quite different. Thus at a p_H of 7·4 which is a normal p_H for reduced arterial blood, the log K for Barcroft's blood is $\bar{4}$·575, for Parsons' lactic acid blood it is $\bar{4}$·295. The few determinations carried out on Parsons' normal blood show that the log K-p_H relation is the same as for the lactic acid blood. The difference between these two lines is far outside the experimental error; it is to be partly accounted for by the fact that the p_H values obtained by Peters for Barcroft's blood are appreciably more acid than those obtained by Parsons working with his own blood. But Peters was more concerned with the differences in p_H produced

in blood under various physiological conditions than with the absolute value of the p_H at any time, and does not claim to have established this absolute value more than approximately in any given case. The average difference between Peters' result at any given pressure of CO_2 and that obtained by Parsons at the same carbon dioxide tension is about 0·1 in the value of the p_H, which is approximately half the difference between the p_H values corresponding to a given value of log K on the Peters-Barcroft curve and that found by Donegan and Parsons [1919]. It is to be noted, however, that Hasselbalch [1916] obtains values for normal blood which coincide with the original Peters-Barcroft curve, but he used p_H values calculated from the carbon dioxide content of the whole blood.

The log K-p_H relationship was tested on 16 cases (see Table I) and the results may be compared with Parsons' lactic acid blood curve in which the log K at $p_H = 7{\cdot}4$ is $\bar{4}{\cdot}295$ and with the P.-B. curve in which the log K at $p_H = 7{\cdot}4$ is $\bar{4}{\cdot}575$.

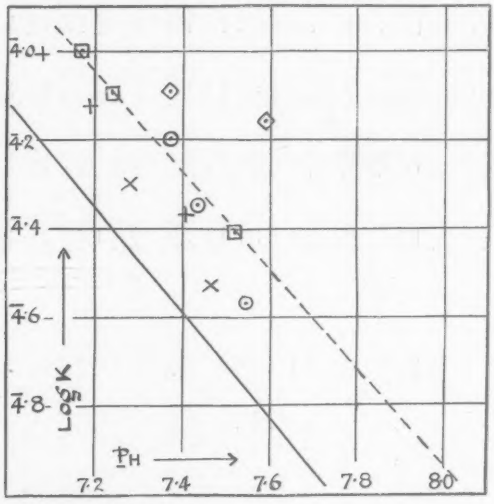

Fig. 1. Log K-p_H curves for typical cases:

+ Case 4 (azotaemic nephritis). ◊ Case 11 (cardio-renal).
⊡ „ 6 (polycythaemia). ⊙ „ 12 (mitral stenosis).
× „ 10 (diabetes). —— P.B. line.
---- Log K-p_H curve obtained with Parsons' blood to which ·04 % of lactic acid had been added.

Some typical examples of the relationship are shown in Fig. 1. In cases 4, 6 and 10 the lines joining the points are parallel with the curve for Parsons' blood and the P.-B. line, while in cases 11 and 12 there is no parallelism. Summarising our results, out of 21 examinations on 14 cases, there are six in which there is no parallelism, whereas in 18 the parallelism is fairly close.

Table I.

Case no.	Age	Disease	Date	Alv. CO_2 mm.	p_H at alv. CO_2 (reduced blood)	p_H at 40 mm. (reduced blood)	Fixed CO_2 at 40 mm. (reduced blood)	Buffer value: red blood	Buffer value: oxyg. blood	O_2 capacity	Average difference: p_H (calc. from blood-true plasma CO_2 curve) − p_H observed	Average difference: p_H (calc. from Warburg) − p_H observed	log K at $p_H = 7{\cdot}4$
5	36	Normal	June 5, '18	(39·4 May 26)	7·34	7·34	43·8	—	4·0 (3)	17·8	−0·033	−0·045	$\bar{4}{\cdot}28$
		,, (after oxygen chamber)	,, 10, '18	(34·9 June 11)	7·34	7·29	43·8	3·3 (4)	4·4 (4)	17·6	+0·039	+0·029	$\bar{4}{\cdot}295$
15	37	Tabes with myatrophy	Sept. 24, '19[1]	39·4	7·375	7·37	40·0	11·2 (2)	—	—	−0·088	−0·103	$\bar{4}{\cdot}35$[2]
1	61	Uraemic coma	Feb. 4, '18	9·6	—	6·72	—	—	1·1 (2)	10·2	—	—	$\bar{4}{\cdot}23$
4	32	Azotaemic nephritis	Mar. 13, '18	34	7·39	7·34	40·3	5·3 (2)	7·2 (2)	22·0	−0·053	−0·048	$\bar{4}{\cdot}44$ (extrap.)
		,, ,,		—	—	—	40·5	—	—	—	+0·01	+0·003	—
		,, ,,	June 24, '18	(36 June 11)	7·26	7·23	39·8	3·95 (4)	3·8 (3)	15·9	+0·057	+0·037	$\bar{4}{\cdot}35$
		,, ,, (after O_2 chamber)	,, 29, '18	(32·7 July 1)	7·31	7·22	42·3	7·3 (4)	2·5 (3)	16·5	+0·08	+0·068	$\bar{4}{\cdot}35$
8	19	Tubal nephritis, lipaemia	Dec. 18, '18	34·9	7·33	7·29	42·3 (extrap.)	—	—	17·8	−0·01	−0·005	$\bar{4}{\cdot}33$[2]
		,, ,,	Jan. 28, '19	34·2	7·34	7·295	43·8	—	8·4 (2)	17·4	+0·03	+0·02	$\bar{4}{\cdot}42$
		,, ,,	,,	—	—	—	40·5	—	—	—	−0·025	−0·053	—
2	56	Cardio-renal	Feb. 12, '18	32·0	—	7·335	45·3	6·1 (3)	—	15·2	0·0	−0·015	$\bar{4}{\cdot}29$
16	55	,,	Nov. 4, '19	29·8	—	7·36	41·6	5·9 (2)	—	24·5	−0·065	−0·05	$\bar{4}{\cdot}34$
11	65	,,	Feb. 7, '19	23·5	—	7·35	44·3	7·5 (2)	—	16·5	−0·008	−0·023	$\bar{4}{\cdot}10$[2]
17	56	Myocardial, emphysema	,, 25, '20	31·2	—	7·36	—	—	—	—	—	—	—
12	21	Mitral disease	,, 14, '19	32·3	7·42	7·37	50·0	9·9 (3)	—	12·6	+0·025	+0·002	$\bar{4}{\cdot}295$[2]
14	14	,,	July 14, '19	35·1	7·50	7·46	—	—	—	22·2	—	—	$\bar{4}{\cdot}19$[3]
7	27	,, pregnancy	Dec. 11, '18	28·1	7·35	7·26	39·3	3·3 (2)	—	16·7	+0·025	+0·008	$\bar{4}{\cdot}15$ (extrap.)
6	46	Erythraemia	Aug. 3, '18	(37·5 July 27)	7·27	7·25	33·6	—	7·3 (3)	30·5	+0·005	+0·013	$\bar{4}{\cdot}27$
		,, (after O_2 chamber)	,, 8, '18	—	7·37	7·35	39·3	—	8·8 (3)	30·2	−0·031	+0·005	$\bar{4}{\cdot}27$
3	42	Diabetes	Feb. 27, '18[1]	36·9	—	7·25	34·3	3·6 (2)	7·9 (2)	18·5	−0·048	−0·06	$\bar{4}{\cdot}29$[3]
		,,	Mar. 4, '18	43·2	7·38	7·39	43·3	—	—	—	−0·08	−0·08	—
10	20	,, Ketosis	Jan. 21, '19	35·3	7·32	7·295	36·8	5·1 (2)	8·2 (2)	19·6	−0·025	−0·038	$\bar{4}{\cdot}45$
		,, ,,	,,	—	—	—	—	—	—	—	−0·038	−0·068	—
9	18	,, ,,	Jan. 14, '19	23·2	7·27	7·14	28·8	3·8 (3)	—	17·0	+0·04	+0·004	$\bar{4}{\cdot}32$
		,, ,,	,,	—	—	—	—	—	—	—	+0·055	+0·01	—
		,, ,,	Feb. 4, '19[1]	16·3	7·23	7·04	—	—	—	18·0	+0·113	+0·04	$\bar{4}{\cdot}85$[2] (extrap.)
		,, ,,	,, 11, '19	19·6	7·32	7·15	29·8	4·7 (3)	—	18·1	+0·073	+0·042	$\bar{4}{\cdot}27$[2]
13	42	,, ,,	June 2, '19	24·5	7·56	7·39	—	—	—	—	—	—	—
		,, ,,	Oct. 21, '19	21·8	7·435	7·265	—	—	—	—	—	—	$\bar{4}{\cdot}64$
		Parson's lactic acid blood	—	—	—	—	—	—	—	—	—	—	$\bar{4}{\cdot}295$
		P.-B. curve	—	—	—	—	—	—	—	—	—	—	$\bar{4}{\cdot}575$

[1] Blood kept in ice overnight.

[2] In this case the log K-p_H relation is not parallel to that of Parsons' blood.

[3] Only one point available for log K-p_H relation.

There was no parallelism in a case of tabes (15) where the blood had been kept overnight, but had not apparently developed much acid; in a cardio-renal case (11); in tubal nephritis (8) on one occasion, but not on another; in mitral stenosis (12); in diabetic ketosis (9) on two occasions out of three, on one of which the blood had been kept, and on these two occasions the lines were not parallel to each other. It may be said that there is usually parallelism, but we cannot find any reasons for the exceptions.

In Table I (last column) we have defined the relationship by the value of log K at p_H 7·4. Analysing these 23 results (two of which are rather doubtful, as only single log K determinations were made) we find that they group themselves roughly about the curve for Parsons' blood. Thus eight results are greater than $\bar{4}$·34 and eight are less than $\bar{4}$·28, while seven are intermediate and the mean of these seven is $\bar{4}$·30, which is practically the same as for Parsons' blood ($\bar{4}$·295).

In cardio-renal disease and mitral disease with heart failure the log K at p_H 7·4 tends to be low, the mean value for cases 2, 16, 11, 12, 14 and 7 being $\bar{4}$·23. This may have some bearing on Lewis, Ryffel, Wolf, Cotton and Barcroft's [1913] determinations of log K in cases of breathlessness. These authors found that the log K was low and considered that this was due to the presence of fixed acid in the blood in abnormal amounts, which produced an acidaemia. In Campbell, Hunt and Poulton's [1923] paper it has been pointed out that the fixed CO_2 of the blood is hardly diminished at all in these cases. Our present results suggest that there is a distinct tendency in cardiac cases for the log K to be low when compared with the p_H. This might possibly explain Lewis and Barcroft's findings; for, out of 13 determinations on the special cases showing meionexy in their paper there are only five in which the p_H at 40 mm. would be more acid than 7·40, supposing that log K was $\bar{4}$·10 at p_H 7·40, as in our case of myocardial degeneration (case 11). In making this estimate we have calculated log K at 40 mm. CO_2 assuming that alteration of log K with CO_2 pressure was the same as in Parsons' lactic blood.

In three cases of diabetes with much ketosis log K at p_H 7·4 tended to be greater than in Parsons' blood, sometimes reaching the P.-B. curve, as follows: case 9 (lipaemia) $\bar{4}$·32 on the first occasion; $\bar{4}$·85, *i.e.* beyond the P.-B. curve on the second occasion when the blood was kept overnight; $\bar{4}$·27 on the third occasion; case 10, $\bar{4}$·45; case 13, $\bar{4}$·64. In order to draw conclusions as to the p_H of blood from determinations of K in six cases of diabetes verging on coma or actually comatose, Poulton [1915] used the P.-B. curve for his calculations of p_H from log K. Our present results indicate that this possibly gave correct results at any rate in some of the cases.

In five cases we have determined the log K-p_H relation on more than one occasion. In cases 4 and 6 (azotaemic nephritis, and erythraemia) there was no alteration before and after treatment in the oxygen chamber; but in case 4 log K at p_H 7·4 changed from $\bar{4}$·44 to $\bar{4}$·35 in three months, while the fixed acid in the blood increased considerably. On the other hand in case 5 (normal)

the fixed acid apparently increased during oxygen treatment but there was no alteration in log K. There was some slight alteration in the slope of the log K-p_H curve and of the log K value at p_H 7·4 in case 8 (tubal nephritis, lipaemia) after six weeks. Case 9 (diabetes with a marked ketosis) showed great changes in the relation within the space of a month.

It is of course unsafe to generalise on a few cases; but our results would appear to suggest that in diabetes with severe ketosis log K tends to be rather high for a given value of p_H. On the other hand in cardiac disease with circulatory failure, including valvular disease and myocardial degeneration, log K tends to be rather low.

3. The comparison of the p_H of blood calculated from the CO_2 of the blood with the p_H determined by the hydrogen electrode.

It has been pointed out by Parsons [1917] that the p_H of blood means the p_H of the "true plasma." Consequently the most direct way of comparing Hasselbalch's CO_2 method with the hydrogen electrode would be to determine at a given CO_2 pressure the CO_2 content of the true plasma and from this to calculate the p_H by means of the formula [see Joffe and Poulton, 1920], and also to measure the p_H of the same specimen directly by the hydrogen electrode. In the case of reduced blood it is unnecessary to use the "true plasma" for the electrode measurement, as the same results are obtained with whole blood.

We have made determinations of this kind in two cases of myocardial degeneration (Figs. 2 and 3). In case 16 the p_H (observed) of the oxygenated plasma was less than that of the reduced blood as would be expected from Parsons' work [1917]. The calculated values from the CO_2 of the plasma agree fairly well with these results, as shown in Fig. 2. [For some unexplained reason the values calculated from the CO_2 of the blood are much too low in this case.] In case 17 (Fig. 3) we have five calculations from the CO_2 of the plasma, and two calculations from the CO_2 of the blood, but, unfortunately, only two values for the observed p_H. The correspondence between them is fair. Further observations of a similar nature would be desirable before it would be possible to say that the truth of this proposition had been conclusively demonstrated.

In case 13 (diabetes with ketosis) we have determinations of p_H both for the reduced blood and oxygenated true plasma. Parsons found that the average difference between the p_H of his oxygenated and reduced blood was 0·038. The differences in the case of the patient's blood were 0·03 and 0·035, agreeing fairly well with Parsons' figures. This difference of course depends primarily on the fact that oxyhaemoglobin is a stronger acid than reduced haemoglobin; and it is satisfactory to be able to conclude that in a pathological state such as diabetes the same theory also holds good.

In all our calculations we have used the Hasselbalch formula [1916] with a constant value of 6·096 for p_{K_1}, according to Warburg's recommendation

[1922, p. 207]. In calculating the free CO_2 we have used Bohr's solubility coefficient for CO_2 in serum, viz. 0·541 [Hasselbalch, 1916].

The true significance of the CO_2 content of true plasma had not been realised during the earlier part of the work, and although we made many determinations of the CO_2 content of whole blood the CO_2 of the "true plasma" was not determined. However, Campbell, Hunt and Poulton [1923] have

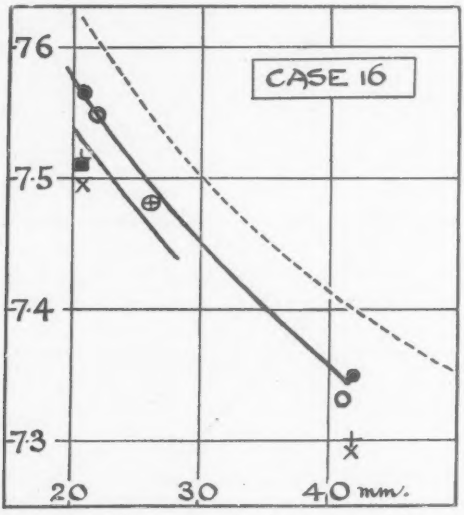

Fig. 2. The dotted line represents the normal relation between p_H and CO_2 tension in the reduced blood of T. R. P.

The points indicate values of this relationship in the blood of case 16, obtained as follows:

● By direct electrometric measurements on the completely reduced blood (T. R. P.).

× By calculation from the CO_2 content of the completely reduced blood (T. R. P.) using the blood-true plasma CO_2 curve.

+ By calculation from the CO_2 content of the completely reduced blood, using Warburg's method.

○ By calculation from the CO_2 content of the true plasma of the partly oxygenated blood (E. P. P.).

■ By electrometric measurement on the true plasma of the fully oxygenated blood (T. R. P.).

⊕ By calculation from the CO_2 content of the true plasma of the fully oxygenated blood (E. P. P.).

determined the CO_2 both in whole blood and in the corresponding true plasma in quite a number of pathological conditions. In Fig. 1 of their paper, where these two values are plotted at right angles to one another, the results fall on a straight line, provided the haemoglobin content of the blood is about normal. Using their diagram we have calculated the CO_2 of the true plasma corresponding to our determinations in whole blood, and these plasma results have then been used for calculating the p_H by Hasselbalch's formula. This has been carried out for all our determinations of CO_2 in blood. It has been thought advisable to make one small correction. Campbell, Hunt and Poulton's curve

applies only to oxygenated blood, while we are dealing here almost entirely with reduced blood. Now Joffe and Poulton found that the difference between the CO_2 content of blood and that of its true plasma was slightly less, if the blood was reduced, than if it was oxygenated. This difference was about 1·7 cc. % and this has been subtracted from the plasma figures obtained from Campbell and Poulton's curve.

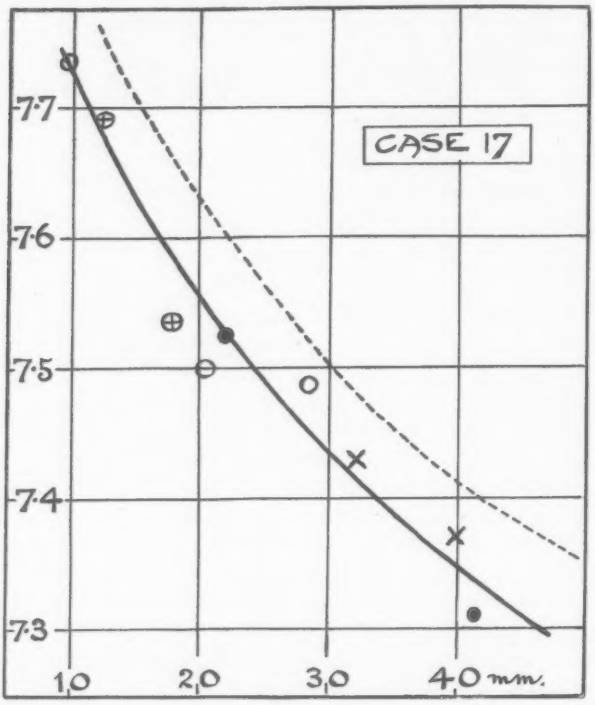

Fig. 3. The dotted line represents the normal relation between p_H and CO_2 tension in the reduced blood of T. R. P.

The points indicate values of this relationship in the blood of case 17, obtained as follows:

● By direct electrometric measurement on the completely reduced blood, and subtraction of 0·038 from each observed value of p_H, in order to bring the readings into comparison with the calculations made on the oxygenated blood.

○ By calculation from the CO_2 content of the true plasma of oxygenated oxalated blood (E. P. P.).

⊕ By calculation from the CO_2 content of the true plasma of oxygenated defibrinated blood (E. P. P.).

× By calculation from the CO_2 content of the whole oxygenated blood (E. P. P.), examined a week later.

In addition to CO_2 determinations on fully reduced blood by T. R. P. with the differential apparatus, in some cases the CO_2 was determined by E.P.P. with Van Slyke's apparatus using the technique described [Joffe and Poulton, 1920]. Sometimes fully oxygenated blood was used and sometimes partly oxygenated

blood. We have calculated the p_H for these results also, but where we are dealing with fully oxygenated blood we have added 0·038 to the result, which is the difference in p_H found by Parsons [1917] between oxygenated and reduced blood, in order to make the results comparable with the p_H measurements on reduced blood. When the blood was partly oxygenated a correspondingly smaller figure was added. These results of E. P. P.'s form a useful check on T. R. P.'s results especially as the methods used are quite different. On the whole the agreement is good.

We have used the same blood-true plasma CO_2 relation in calculating the p_H in our case of polycythaemia, because there is evidence that this relation is unchanged in polycythaemia, where the fixed acid of the blood is increased.

There is one other point. Campbell, Hunt and Poulton constructed their blood-plasma CO_2 relation for oxalated blood, while we are dealing here almost exclusively with defibrinated blood. Thus we have neglected any slight alteration there may be in the distribution of CO_2 between corpuscles and plasma, as the result of adding 0·5 % potassium oxalate.

The p_H values obtained by calculation and by actual experiment are given in detail in Table IV.

In Table II the differences between the values have been collected and it will be seen that out of the 57 calculations 71·9 % are not further from the observed values than 0·05. In Table I the average differences of the several determinations in each case are stated. When the fixed acid of the blood is increased, as indicated by the low value of p_H at 40 mm., there is a decided tendency for the p_H calculated from the blood-true plasma CO_2 curve to be higher than the observed value. This is shown in the last two experiments in case 4, in case 7 and in case 9. In the last case when the fixed acid in the blood due to keeping had increased greatly, the difference was 0·113, which is much the largest we found.

Table II.

Total No. of observations Percentage	No. of observations showing a difference between p_H (calculated) and p_H (observed) not greater than				Greater than 0·1
	0·01	0·03	0·05	0·1	
Blood-true plasma CO_2 curve used:					
57	14	26	41	53	4
%	24·6	45·6	71·9	93	
39*	13	23	31	38	1
%	33·3	59	79·5	97·4	
Warburg's method used:					
57	15	31	45	55	2
%	26·3	54·4	79	96·5	
39*	10	22	32	38	1
%	25·6	56·4	82·1	97·4	

* After deducting the cases in which there was a considerable increase of fixed acid, but not case 6.

On the other hand in case 3, where acid had accumulated owing to keeping, the difference was − 0·048; but the difference was − 0·06 for the same case when there was no acid, *i.e.* the alteration due to acid was in the same direction as in the other cases.

We think it possible that the relationship between the CO_2 in the corpuscles and plasma is altered by the addition of acid, so that the plasma, owing to the greater neutralisation of sodium bicarbonate contains relatively less CO_2 than the corpuscles [see Campbell, Hunt and Poulton, 1923, Section 3]. This would account for the difference. It is clear that these cases with increased fixed acid should be omitted from our test of the accuracy of the Hasselbalch formula applied to the plasma. This leaves 39 determinations and Table II shows a considerable improvement in the agreement, 79·5 % of the calculated p_H values not being further from the observed p_H than 0·05. Seven of the calculated p_H values coincide with the observed p_H, 20 are too small and 12 are too large.

Warburg[1] has recently described a modification of the Hasselbalch formula by means of which it is possible to calculate the p_H of blood (*i.e.* of the true plasma) from the CO_2 content of the blood and the CO_2 pressure, provided the oxygen capacity of the blood is known.

The oxygen capacities were obtained by Parsons during his log K determinations. They were carried out with various Barcroft differential apparatus in which (except for case 15) the oxygen capacity of Parsons' own blood had also been obtained. In making the calculations the value for Parsons' blood was taken at 18·5 vols. %; but it had not been determined. The haemoglobinometer value was 98 % (Price Jones).

As some of the examples in Warburg's paper are not quite accurately calculated and as the method is a valuable one, we think it best to give a fresh example of the calculation and Dr Warburg has kindly agreed to this.

Example. Case 8, on Jan. 28, 1919.

Partly oxygenated blood (% saturation = 60·3) contained 41·2 cc. CO_2 % at 41·7 mm. CO_2 pressure. It is required to find the p_H of this blood. The oxygen capacity (*i.e.* combined oxygen) of the blood was 17·4 vols. %.

In order to calculate the volume of free CO_2, Warburg calculates the absorption coefficient Ψ by equation 128 and multiplies the pressure of CO_2 by the factor $\frac{0{\cdot}5554}{7{\cdot}6} \times \Psi$. We have shortened the process considerably by calculating a graph of the blood, which relates the oxygen capacity of the blood as abscissa with the above factor as ordinate directly. The graph is a straight line, drawn through two points such that an oxygen capacity of 1·5 vols. % corresponds to the factor 0·0709 and an oxygen capacity of 37·5 vols. corresponds to the factor 0·0620 (see Fig. 4). In the present example the factor corresponding to 17·4 vols. % oxygen is 0·0670.

[1] Dr Warburg has pointed out a mis-print to us in equation 206 [1922, p. 327] which should read $x_1 = 100 -$ the fraction.

The free CO_2 is $41{\cdot}7 \times 0{\cdot}067 = 2{\cdot}794$.

Now p_H (uncorrected) $= 6{\cdot}155 + \frac{\log \text{combined } CO_2}{\log \text{free } CO_2}$ [see Warburg, 1922, p. 202]

$$= 6{\cdot}155 + \frac{\log (41{\cdot}2 - 2{\cdot}794)}{\log 2{\cdot}794}$$

$$= 7{\cdot}293.$$

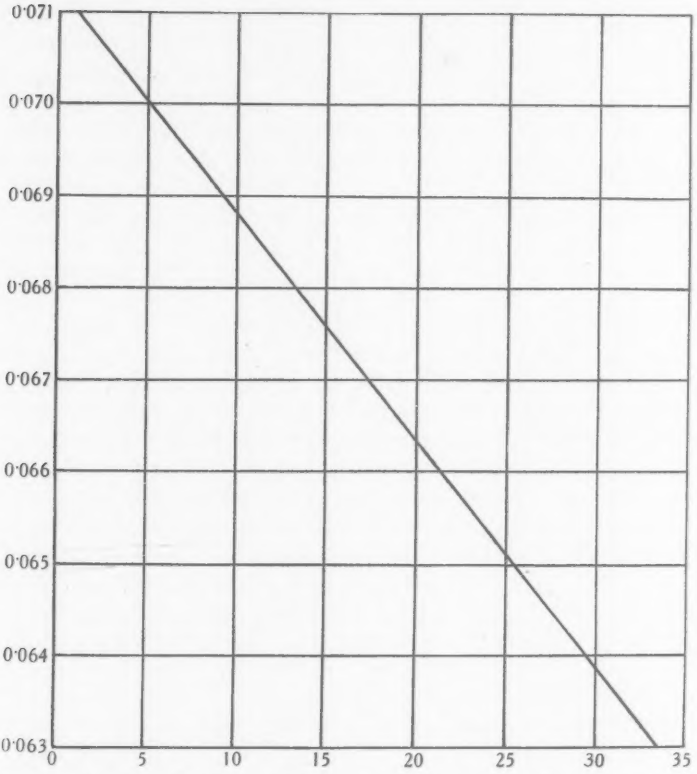

Fig. 4. Calculation of p_H (Warburg). Ordinate, values of F in equation—Free CO_2 (cc. per 100 cc. blood) $= F \times CO_2$ partial pressure (mm. Hg.). Abscissa, oxygen capacity of blood (cc. per 100 cc.).

The correction is obtained as follows: a value of p_H depending on the percentage saturation of the blood (60·3) is first calculated, viz.

$$7{\cdot}293 - \frac{100 - 60{\cdot}3}{100} \times 0{\cdot}40$$

$$= 7{\cdot}13.$$

The correction corresponding to this (7·13) is obtained from Fig. 10 of Warburg's paper corresponding to 17·4 % oxygen. This is + 0·005. This is added to the original figure 7·293, so that the p_H is 7·298. Warburg has expressed his values

of p_H on Bjerrum's scale [see p. 167 of his paper]. Hence 0·048 must be deducted, so that the finally corrected p_H of the blood is 7·25 on the Sörensen scale. We have made an addition of 0·025 to this figure in this particular case, so as to make the result comparable with the observed p_H which was carried out on reduced blood.

We have calculated all our available results by Warburg's method and their accuracy can be compared with those calculated from the blood-true plasma CO_2 curve. A summary of the 57 results in Table II shows that the accuracy of Warburg's method is rather greater. This is because in cases 4, 7 and 9, in which the fixed acid of the blood was increased, the error is not so great by this method of calculation, as can be seen in Table I. After deducting these cases the accuracy of the two methods in the 39 remaining determinations is very much the same (see Table II). Of the 39 values calculated by Warburg's method 26 were less and 11 were greater than the observed p_H.

In this connection case 6 (erythraemia) is of special interest (Table I). On Aug. 8th after oxygen treatment the fixed acid in the blood was about normal. Under these circumstances Warburg's method of calculation would be expected to give a more accurate result, while the result calculated from the blood-true plasma CO_2 curve would be too low because with increase of blood cells there would be relatively more CO_2 in the plasma compared with the blood [see Campbell, Hunt and Poulton, 1923, Section 9]. The Warburg figure is 0·005 too large and the figure from the curve is 0·031 too small. Before treatment with oxygen the blood contained excess of fixed acid. This causes a relative diminution in the CO_2 of the plasma which neutralises the previous error in the calculation from the blood-true plasma CO_2 curve. On the other hand this makes the Warburg calculation too large. In accordance with this we find that on Aug. 3rd the former value was the more accurate, being only 0·005 too large, while the Warburg calculation was 0·013 too large.

In case 12 the oxygen capacity was rather low, without increase of fixed acid. This would make no difference to the Warburg figure but would make the figure calculated from the curve too high. In accordance with this we find that the former was only 0·002 too high, while the latter was 0·025 too high.

The conclusion we would draw is that when the haemoglobin content is about normal both methods of calculation from the CO_2 content of the blood are available; where the haemoglobin is distinctly too high or too low and there is no increase of fixed acid or base, Warburg's method is the better; where there is increase or diminution in fixed acid, it is necessary to determine the CO_2 in the true plasma and make the calculation from this by Hasselbalch's formula using a constant value of 6·096 for p_{K_1}.

4. The p_H of reduced blood at 40 mm. CO_2 pressure.

The method which has been widely adopted for obtaining a measure of the fixed acid in the blood was described by Van Slyke and Cullen [1917],

viz. determining the alkali reserve of separated plasma (the CO_2 combined at 40 mm.). A better plan is to determine the CO_2 combined at 40 mm. in whole blood [Joffe and Poulton, 1920]. It is permissible in most cases to draw conclusions as to the fixed acidity of the blood from these measurements, providing that the blood has about a normal haemoglobin content. It is still better to determine the CO_2 combined in the true plasma, as this gives a measure of the amount of fixed acid present, irrespective of the haemoglobin content of the blood. Undoubtedly the best method of measuring variations in fixed acidity is to determine the reaction of the blood by some direct method at a known pressure of CO_2 such as 40 mm. For this purpose we have used the hydrogen electrode, and figures have been obtained for 17 cases, some of them being examined on several occasions.

The normal limits for the p_H of reduced blood at 40 mm. CO_2 pressure have been determined by Barcroft and his co-workers [1922] by observations on nine different subjects. The values ranged from 7·38 to 7·47. Parsons' blood at one time gave a value of 7·36 [Donegan and Parsons, 1919], so that we may regard 7·36–7·47 as the normal limits.

In our series of 17 cases results between normal limits were obtained in seven and six of them lay between 7·36 and 7·39, so that they were on the acid side of Barcroft and his co-workers' mean value. The seven cases comprised two each of diabetes, mitral disease, and myocardial disease, and one normal (15) in which the blood had been kept overnight. In the other normal case (5) the value was 7·34, which is slightly acid; but for some unexplained reason the fixed acidity was still further increased after treatment in an oxygen chamber.

Cases with evidence of heart failure also gave mostly normal results. These included two cases of mitral stenosis—case 12, $p_H = 7·37$, and case 14, $p_H = 7·46$, and four cases of myocardial degeneration which were clinically similar to the cases described by Lewis and his colleagues [1913]. They were: case 2, 7·335; case 11, 7·35; case 16, 7·36; and case 17, 7·36. These results agree with those of Campbell, Hunt and Poulton [1923] and indicate that there is no large amount of fixed acid present in the blood, as has been suggested.

In one case of diabetes with very little ketosis (case 3) the p_H was normal, 7·39. In case 13 where the ketosis was not marked on one occasion, due to treatment, the p_H was 7·39, though on a second occasion when ketosis had increased the value of 7·265 was obtained showing considerable increase in the fixed acid of the blood. Case 4, azotaemic nephritis, gave a value of 7·34 on one occasion, but on a second occasion three months later the value was 7·23 before and 7·22 after oxygen, showing a considerable increase in the fixed acid of the blood.

Six of our cases showed a marked increase in the fixed acidity. One of these (case 1) was a case of uraemia following septic pyelonephritis. The p_H of the blood while the patient was unconscious shortly before death was 6·72, the blood being actually acid, but it had been kept 16 hours longer than usual

although in ice all the time. The other was a case of parenchymatous nephritis with oedema, 7·29 and 7·295 on two occasions.

Two cases of diabetes with ketosis showed increase in the fixed acidity. In case 10 this was slight, the p_H being 7·295. In case 9 values of 7·14, 7·04 and 7·15 were obtained. The middle one of these determinations—7·04—was done when the patient was approaching coma, but the blood had been kept in ice overnight.

In one case of mitral stenosis (case 7) with pregnancy the p_H was 7·26, the acid value being probably accounted for by the pregnancy (see Hasselbalch and Gammeltoft, 1915].

In case 6 (erythraemia with splenomegaly) the p_H was 7·25, showing increase in fixed acidity. The interesting point was that after treatment with oxygen the p_H rose to 7·35 a normal figure, so that the acid, whatever it was, was abolished by oxygen. Dr Price Jones found that the condition of the blood remained unaltered (Table III). This suggests that the polycythaemia was not secondary to want of oxygen [see Weber, 1921].

In Table I the fixed CO_2 blood at 40 mm. is also given. On the whole the values diminish with the p_H, as would be expected; but there are surprising exceptions, *e.g.* case 4, where the CO_2 remained much the same, while the fixed acid, as indicated by the p_H, had increased considerably.

Table III.

Case	Date	Red cells $\times 10^6$ white cells per cu. mm.	Hb. per cent. colour index	Mean diameter of red cells	Differential Count. per cu. mm.				
					Polymorphs	Lymphocytes	Hyalines	Eosinophils	Mast cells
4	24. vi. 18	5·41	86	7·44	6922	6306	1050	293	117
		14,666	0·79	—	—	—	—	—	—
	29. vi. 18	4·136	88	7·55	3793	3863	835	157	52
	(after O_2 chamber)	8,700	1·07	—	—	—	—	—	—
5	5. vi. 18	4·856	94	7·56	7330	2415	588	147	21
		10,500	0·96	—	—	—	—	—	—
	10. vi. 18	4·86	98	7·34	6271	4680	468	281	0
	(after O_2 chamber)	11,700	1·01	—	—	—	—	—	—
6	3. viii. 18	8·346	158	6·87	4350	390	150	100	10
		5,000	0·95	—	(10 normoblasts, 10 myeloblasts)				
	9. viii. 18	8·646	164	6·91	5504	647	251	145	53
	(after O_2 chamber)	6,600	0·95	—	(40 normoblasts)				

5. p_H OF REDUCED BLOOD AT ALVEOLAR CO_2 PRESSURE.

This calculation has often been made in order to express the p_H of the arterial blood on the assumption that the alveolar and arterial CO_2 pressures are identical. In the paper by Campbell, Hunt and Poulton [1923] it is shown that this approximately holds in people who are not breathless, and in anaemia and erythraemia, while in cases of mitral stenosis there is sometimes fairly good correspondence and sometimes not. However in breathless cases where there was evidence of pulmonary disease the alveolar samples gave results very much below the arterial CO_2 pressure. Many of these cases might be classified as cardio-renal, although there was evidence that the lungs were

also affected. On this account we have not calculated the p_H at alveolar CO_2 pressure for cases 2, 16, 11 and 17, as we think the results would be misleading.

The figures in column 5 (Table I) are for fully reduced blood and it is necessary to deduct about 0·04 to make these strictly comparable to the figures for arterial blood. The results, as is the case with all figures hitherto published, may err somewhat on the acid side, as the observations were made before Lovatt Evans' work on glycolysis was published. This subject is dealt with fully elsewhere [Campbell, Hunt and Poulton, 1923].

Approximately normal results were obtained in cases 5, 15 (normals) and in case 4 on March 4th while the p_H fell somewhat as the fixed acid in the blood was increased later and in case 8 (tubal nephritis). In case 6 (erythraemia) the p_H was 7·27 which is low and this was fairly close to the arterial value for case 13 of the next paper. In case 12 (mitral stenosis) the value 7·42 was quite close to the arterial value 7·395 of the same patient in Campbell, Hunt and Poulton's paper (case 9). In case 27 the value was normal showing that the increase of fixed acid due to pregnancy had been approximately compensated. In case 1 (uraemia) there was great acidaemia, but the blood had been kept longer than normal. Still, taken in conjunction with case 33 of Campbell, Hunt and Poulton's paper there can be little doubt that acidaemia is present in uraemic coma, but evidence that the coma is not itself due to the acidaemia is given in their paper.

In the ketosis of severe diabetes there are reasons [Campbell, Hunt and Poulton, 1923] for believing that the alveolar samples may give too low values for the arterial CO_2. If this is the case, the arterial p_H would probably lie somewhere between the value at alveolar CO_2 pressure and the value at 40 mm. This would suggest that there was a measurable amount of acidaemia in case 9 who was verging on a state of coma when the examinations were made. Sonne and Jarlöv [1918] found the same thing by determinations of the CO_2 of blood at 40 mm. Poulton [1918] also came to this conclusion from log K determinations. However it does not follow that this acidaemia is itself the cause of diabetic coma. Cases of emphysema with myocardial changes are described by Campbell, Hunt and Poulton, and in these cases there was for months some degree of acidaemia due to CO_2 without any symptoms of drowsiness. However it must be admitted that in the case of diabetes further observations with analysis of the arterial blood are necessary before conclusions can be drawn as to whether acidaemia plays any part in the symptomatology of coma. On other grounds this is unlikely [Poulton, 1918].

6. The buffer value of blood.

Following Barcroft, Bock, Hill, Parsons, Parsons and Shoji [1922] we have considered that buffer value should be expressed as a change in c_H and we define it as the percentage volume of CO_2 it is necessary to add to blood within the physiological range, to increase the c_H by 1×10^{-8}.

The buffer value of blood is difficult to estimate because if only a few p_H

and CO_2 points are determined; a slight error in the determination will make a considerable change in the values obtained. In many of our cases we have not only the CO_2 volumes determined on the same samples of reduced blood which were used for the p_H measurements, but we have also CO_2 measurements on the partly oxygenated blood used for the log K determinations. These latter CO_2 determinations have not so far been employed, and are not given in the protocols, because the constant of the differential apparatus used was not known with the same degree of accuracy as that of the apparatus used for the reduced blood. However the results are comparable among themselves, so that they can be used for obtaining the buffer value. In Table I calculations from the CO_2 of both reduced and partly reduced oxygenated blood are given and the number of points available for the calculation are put in brackets after the value.

The most striking fact arising out of these results is the effect that increasing the fixed acid of the blood has in diminishing the buffer value. Taking cases 3, 4, 7 and 9, when the p_H at 40 mm. was not above 7·26 the average buffer value was 4·54, while taking the cases where the p_H at 40 mm. was above 7·33, the average buffer value was 7·0.

Anaemia also diminishes buffer value; but this can hardly be of much importance in these cases because the average oxygen capacity was not very different in the two groups, being 17·1 in the first and 19·1 in the second.

The remarkably low value of 1·1 was obtained in case 1 (uraemia), where the fixed acid was very greatly increased, and there was also considerable anaemia.

In conclusion, we should like to thank Dr Price Jones for allowing us to quote some of his blood findings, and Dr W. N. Hurtley for kindly analysing the urine in case 13.

The expenses of this investigation were defrayed by Government Grants from the Royal Society and a maintenance grant to one of us (T. R. P.) from the Medical Research Council.

7. Conclusions.

(1) The log K-p_H relationship was a straight line, in most cases parallel to the P.-B. line and to the line for Parsons' blood, but it was usually closer to the latter. In cardio-renal disease and mitral disease with heart failure the log K at p_H 7·4 tended to be rather low. It was rather higher in a few cases of diabetes with ketosis.

(2) The p_H can be calculated from the CO_2 content of blood and the CO_2 partial pressure, (*a*) by calculating the "true" plasma CO_2 from the blood—true plasma CO_2 curve given by Campbell, Hunt and Poulton [1923], (*b*) by Warburg's method. The latter method must be used where there is any great increase or diminution in the oxygen capacity of the blood. If there is increase or diminution of CO_2 fixing power, it is essential to determine the CO_2 content of the true plasma and use this for the calculation. In suitable cases 80 % of values were found not to be further away from the observed p_H than 0·05.

(3) A graph has been constructed which should be useful in calculating p_H values by Warburg's method.

(4) A case of erythraemia with splenomegaly, treated for five days in an oxygen chamber, at Cambridge, showed no alteration in the blood picture. There was a marked diminution in the fixed acid of the blood, which was previously abnormally high.

(5) In cases of diabetes with ketosis, in mitral disease with pregnancy, in uraemia and renal disease and erythraemia, the p_H observed at 40 mm. was abnormally low. In other cases normal values were usually obtained.

(6) The buffer value of blood is diminished, as the fixed acid in blood is increased.

8. Clinical Notes on Cases and Protocols.

Case 1. *Male.* Enlarged prostate. Suppurative pyelo-nephritis. Uraemia. Blood urea 0·4 %.

Case 2. *Male.* Myocardial degeneration, nephritis and bronchitis, anasarca, blood urea 0·046 %.

Case 3. *Female.* Diabetes mellitus. Faint Rothera reaction.

Case 4. *Male.* Azotaemic nephritis following streptococcal pneumonia. Blood urea on Oct. 26, Nov. 10, 1917, Feb. 7, March 15, June 12, 1918, respectively 0·2, 0·09, 0·08, 0·05, 0·07 %. B.P. (Mar. 19) 183/110. Uraemia and death in March 1921. Blood urea 0·44 %.

Case 5. *Healthy Male.* Recovery after having suffered in Jan. 1918 from acute nephritis and uraemia with hemiplegia. Blood urea on Jan. 29, Feb. 12, April 10, 25, May 7, respectively 0·23, 0·03, 0·03, 0·03, 0·02 %.

Case 6. *Male.* Erythraemia with enlarged spleen. B.P. 160.

Case 7. *Female.* Mitral stenosis, 4–5 months pregnancy. Some bronchitis.

Case 8. *Male.* Chronic tubal nephritis, lipaemia, oedema. Blood urea 0·02 %.

Case 9. *Male.* Diabetes, ketosis, lipaemia, sepsis. Ammonia index 23·3 %. Coma threatening on Feb. 3 with subsequent recovery. Died a few weeks later.

Case 10. *Male.* Diabetes, ketosis, septic glands.

Case 11. *Male.* Myocardial degeneration, emphysema, pulsus alternans, anasarca, oedema of lungs, albuminuria. Blood urea 0·04 %. Cheyne-Stokes respiration. No cyanosis. Large tender liver.

Case 12. *Female.* Mitral stenosis and regurgitation. Bronchitis. Oedema. Large liver. No cyanosis. (The same as case 9 of Campbell, Hunt and Poulton's paper.)

Case 13. *Female.* Diabetes, ketosis. Given 3 drachms sod. bicarb. daily. June 2, Urine sugar 3·6 %; Ammonia index 8 %; Aceto-acetic acid slight. Oct. 21, ketosis had much increased. Dr W. N. Hurtley of St Bartholomew's Hospital very kindly analysed the urine (184 cc.) collected during the 12 hours, 8 p.m. to 8 a.m., before the blood sample was taken. Aceto-acetic acid was

0·19 %; total 3·51 gm. The β-hydroxybutyric acid was 0·63 %; total 11·6 gm. as determined by rotation and 0·94 %; total 17·3 gm. as determined by titration. The ratio $\frac{\beta\text{-hydroxybutyric acid}}{\text{total acetone bodies}}$, was 82·6 % agreeing with Neubauer's and Kennaway's findings [1914] in severe diabetes.

Case 14. *Female.* Mitral stenosis and regurgitation, large pulsating liver, ascites, right sided dilatation of heart, lips slightly cyanosed, orthopnoea.

Case 15. *Male.* Tabes with myatrophy, taken as a normal control.

Case 16. *Male.* Fibroid heart (650 g.), Atheroma of coronary arteries, Infarcts of spleen and kidneys. Auricular fibrillation. Oedema, periodic breathing and orthopnoea. Distinct cyanosis of face and hands. Blood urea 0·04 %. Slight albuminuria.

Case 17. The same as case 26 [Campbell, Hunt and Poulton, 1923].

Table IV.

		Fully reduced blood					Partly oxygenated blood	
			p_H (calculated)					
Case	Date	CO_2 mm.	p_H (observed)	Bl. t. pl. curve	Warburg	CO_2 cc. %	CO_2 mm.	log K
1.		9·9	6·92	—	—	—	10·5[1]	$\bar{5}$·74
		26·0	6·81	—	—	—	23·7[1]	$\bar{5}$·66
		40·2	6·72	—	—	—	—	—
2.		23·1	7·51	7·51	7·49	39·4	20·1	$\bar{4}$·43
		33·1	7·39	7·39	7·38	44·2	29·0	$\bar{4}$·33
		41·7	7·33	7·33	7·315	49·0	41·4	$\bar{4}$·25
3.	Feb. 27	27·1	7·40	7·34	7·33	31·8	25·3	$\bar{4}$·32
		35·4	7·30	7·265	7·25	35·5	—	—
	Mar. 4	44·3	7·37	7·29	7·29	48·1	—	—
4.	Mar. 13	27·3	7·465	7·39	7·40	36·7	30·4	$\bar{4}$·44
		48·9	7·27	7·24	7·24	47·0	56·4	$\bar{4}$·29
	June 24	2·5	7·84	—	—	8·2	11·6	$\bar{4}$·19
		25·0	7·34	7·435	7·42	36·2	35·7	$\bar{4}$·05
		40·8	7·23	7·265	7·24	40·6	49·2	$\bar{4}$·52
		61·6	7·11	7·15	7·13	48·8	63·3	$\bar{5}$·96
	June 29	2·3	7·96	—	—	5·0	0·5	$\bar{3}$·13
		23·5	7·37	7·42	7·405	33·0	18·6	$\bar{4}$·56
		42·7	7·20	7·31	7·30	47·9	32·9	$\bar{4}$·22
		64·9	7·07	—	—	—	64·5	$\bar{5}$·95
5.	June 5	1·7	8·17	—	—	11·8	11·8	$\bar{4}$·72
		16·8	7·61	7·555	7·54	30·9	26·9	$\bar{4}$·31
		45·6	7·32	7·29	7·285	49·4	38·8	$\bar{4}$·21
		33·2	7·39	7·375	7·37	42·7	52·4	$\bar{4}$·10
	June 10	1·9	8·18	—	—	5·6	10·2	$\bar{4}$·67
		17·9	7·56	7·60	7·605	38·0	20·8	$\bar{4}$·41
		31·0	7·37	7·41	7·395	42·4	41·1	$\bar{4}$·15
		45·3	7·24	7·285	7·28	48·4	57·1	$\bar{4}$·10
		58·9	7·17	7·20	7·175	50·7	55·7	$\bar{4}$·06
6.	Aug. 3	22·2	7·36	—	—	—	—	—
		40·6	7·24	—	—	—	—	—
		60·4	7·13	—	—	—	—	—
	Aug. 8	12·5	7·63	—	—	—	—	—
		38·8	7·36	—	—	—	—	—
		60·7	7·23	—	—	—	—	—
7.		35·0	7·29	7·335	7·315	40·8	31·6	$\bar{4}$·08
		49·8	7·20	7·205	7·19	44·7	45·8	$\bar{4}$·01
8.	Dec. 18	30·0	7·38	7·37	7·375	39·3	24·0	$\bar{4}$·43
		39·6	7·30	—	—	—	38·8	$\bar{5}$·16
		—	—	—	—	—	50·3	$\bar{4}$·93
	Jan. 28	37·5	7·32	7·35	7·34	45·4	16·7	$\bar{4}$·595
		—	—	—	—	—	30·3	$\bar{4}$·44
		—	—	—	—	—	40·0	$\bar{4}$·26

Table IV *continued.*

Case	Date	Fully reduced blood: CO_2 mm.	p_H (observed)	p_H (calculated): Bl. t. pl. curve	p_H (calculated): Warburg	Fully reduced blood: CO_2 cc. %	Partly oxygenated blood: CO_2 cm.	Partly oxygenated blood: log K
9.	Jan. 14	16·0	7·33	7·405	7·36	20·3	16·1	$\bar{4}$·26
		29·3	7·22	7·24	7·205	27·0	31·4	$\bar{4}$·13
		49·1	7·08	7·105	7·07	34·5	—	—
	Feb. 4	15·1	7·245[2]	7·425	7·355	18·8	14·0	$\bar{4}$·496
		14·6	7·25	—	—	—	29·4	$\bar{4}$·14
		29·2	7·105	7·15	7·065	20·1	—	—
	Feb. 11	9·4	7·44	7·555	7·525	16·8	11·2	$\bar{4}$·27
		26·8	7·22	7·26	7·225	25·5	39·2	$\bar{4}$·20
		36·3	7·17	7·235	7·205	33·6	—	—
10.		20·3	7·465	7·465	7·455	31·0	22·1	$\bar{4}$·47
		42·9	7·28	7·23	7·215	40·3	35·4	$\bar{4}$·40
		—	—	—	—	—	43·4	$\bar{4}$·29
11.		16·3	7·59	7·59	7·58	32·8	16·2	$\bar{4}$·16
		41·2	7·345	7·33	7·31	47·5	37·2	$\bar{4}$·09
12.		19·8	7·545	7·59	7·565	39·8	19·5	$\bar{4}$·58
		28·3	7·45	7·48	7·45	44·4	29·5	$\bar{4}$·02
		41·7	7·37	7·37	7·355	53·8	46·7	$\bar{4}$·17
13.	June 2	20·5	7·62	—	—	—	—	—
		28·9	7·52	—	—	—	—	—
		44·3	7·34	—	—	(Oct. 21)	11·7	$\bar{4}$·80
	Oct. 21	20·9	7·45	—	—	—	26·0	$\bar{4}$·64
		39·6	7·27	—	—	—	35·5	$\bar{4}$·57
14.		27·8	7·575	—	—	—	29·9	$\bar{4}$·41
		40·4	7·46	—	—	—	—	—
15.		29·3	7·46	7·325	7·30[3]	32·8	28·2	$\bar{4}$·37
		49·8	7·30	7·26	7·255[3]	50·1	47·7	$\bar{4}$·29
16.		20·7	7·565	7·495	7·515	34·4	21·7	$\bar{4}$·49
		41·7	7·35	7·29	7·30	44·8	41·0	$\bar{4}$·30
17.		21·5	7·56	—	—	—	—	—
		41·5	7·35	—	—	—	—	—
		Experiments on fully oxygenated blood						
4.	Mar. 13	29·8	7·43[4]	7·415	7·42	36·5	—	—
		38·4	7·35[4]	7·385	7·365	42·3	—	—
13.	Oct. 21	21·0	7·415[5]	—	—	—	—	—
		39·3	7·24[5]	—	—	—	—	—
16.	Mar. 13	20·7	7·51[5]	—	—	—	—	—
		Experiments on partly oxygenated blood (Hb. circ. 50 % saturation)						
6.	Aug. 3	16·0	7·41[6]	7·41[7]	7·405[7]	19·1	—	$\bar{4}$·39
		37·2	7·26[6]	7·265[7]	7·28[7]	34·5	—	$\bar{4}$·12
		53·3	7·17[6]	7·18[7]	7·195[7]	42·7	—	$\bar{5}$·98
	Aug. 8	9·9	7·66[6]	7·665[7]	7·705[7]	21·4	—	$\bar{4}$·50
		27·9	7·455[6]	7·385[7]	7·415[7]	33·8	—	$\bar{4}$·36
		43·1	7·335[6]	7·305[7]	7·34[7]	45·0	—	$\bar{4}$·17
		58·4	7·24[6]	7·21[7]	7·24[7]	50·0	—	$\bar{4}$·09
8.	Jan. 28, '19	15·4	7·53[6]	7·475[7]	7·435[7]	21·9	—	—
		41·7	7·285[6]	7·29[7]	7·275[7]	41·2	—	—
9.	Jan. 14, '19	32·6	7·19[6]	7·245[7]	7·20[7]	28·6	—	—
10.		14·8	7·53[6]	7·48[7]	7·435[7]	20·6	—	$\bar{4}$·66
		35·7	7·32[6]	7·295[7]	7·28[7]	35·9	—	$\bar{4}$·327

[1] Corresponding values for CO_2 content were 5·9 and 8·6 cc. %.

[2] By extrapolation.

[3] Assuming haemoglobin content of blood was normal.

[4] Values obtained by interpolation from reduced blood, with 0·038 added, so that they represent p_H of fully oxygenated blood [see Parsons, 1917].

[5] Oxygenated true plasma was used for these determinations.

[6] These values are for reduced blood, obtained by interpolation from the values given earlier in the table.

[7] CO_2 in partly oxygenated blood was used in these calculations, which were subsequently corrected so that they represent values of p_H for reduced blood.

REFERENCES.

Barcroft (1914). The Respiratory Function of the Blood, Cambridge.
Barcroft and Peters (1914). *Phil. Trans. Roy. Soc.* B. **206**, 67.
Barcroft and co-workers (1922). *J. Physiol.* **56**, 157.
Campbell, Hunt and Poulton (1923). *J. Path. Bact.* **26**, 234.
Donegan and Parsons (1919). *J. Physiol.* **52**, 315.
Hasselbalch (1913). *Biochem. Z.* **49**, 451.
—— (1916). *Biochem. Z.* **78**, 112.
—— (1917). *Biochem. Z.* **82**, 282.
Hasselbalch and Gammeltoft (1915). *Biochem. Z.* **68**, 206.
Joffe and Poulton (1920). *J. Physiol.* **54**, 129.
Kennaway (1914). *Biochem. J.* **8**, 355.
Lewis, Ryffel, Wolf, Cotton and Barcroft (1913). *Heart*, **5**, 45.
Parsons (1917). *J. Physiol.* **51**, 440.
Peters (1914). *J. Physiol.* **48**. *Proc.* vii.
Poulton (1915). *J. Physiol.* **50**. *Proc.* i.
—— (1918). (Goulstonian Lectures). *Lancet*, June 29th.
Poulton and Ryffel (1913). *J. Physiol.* **46**. *Proc.* xlvii.
Sonne and Jarlöv (1918). *Arch. klin. Med.* **124**, 379.
Van Slyke and Cullen (1917). *J. Biol. Chem.* **30**, 289.
Warburg (1922). *Biochem. J.* **16**, 153.
Weber (1921). Polycythaemia, H. K. Lewis and Co.

XXXIX. THE EFFECT OF A YEAST EXTRACT ON THE OXYGEN CONSUMPTION OF WASHED FROG MUSCLE.

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(*Received March 15th, 1923.*)

In 1911 Batelli and Stern [1911] showed that if some finely divided mammalian muscle was either allowed to respire until the velocity of its respiration was somewhat diminished or was washed with a little cold water, which produced a similar effect, the addition of an extract of various mammalian tissues made with cold water increased the velocity of the respiration and in some cases restored it to its initial value.

They showed that the effect was temporary, but that a second addition of such a tissue extract produced a second, temporary, increase in the velocity of respiration. They assumed that a substance which they called "Pnein" was present in these extracts. They found that by a short period, 45 minutes, of respiration, the "Pnein" was not destroyed to any great extent.

In 1918 and 1919 Meyerhof working on "acetone-yeast" [1918] and frog muscle [1919, 1] showed that in the case of each of these substances the velocity of respiration could be very considerably reduced by exhaustive washing and could afterwards be restored by the addition of an extract of yeast or of animal tissues made with boiling water ("Koch-saft").

He assumed the presence in these extracts of a substance or a mixture of substances, which accelerated the velocity of respiration, and named it the "Respiration-substance" (Atmungs-körper). He advanced the hypothesis that the "respiration-substance" probably consists of a "co-enzyme to respiration" which is an oxygen-carrier [Meyerhof, 1919, 2] and is identical with the co-enzyme to alcoholic fermentation [Harden and Young, 1906], together with a supply of oxidisable material. He observed that, on warming such an extract for some hours in the air [Meyerhof 1918], a portion of the "respiration-substance" was destroyed.

The experiments described in this paper were undertaken in an attempt to obtain evidence as to the actual existence of a "co-enzyme to respiration" of the type suggested by Meyerhof.

Some finely chopped frog's muscle was washed several times with distilled water. It was then aerated for several hours at the ordinary temperature of

the laboratory whilst suspended in a yeast "Koch-saft." The liquid was then filtered off from the muscle, and used as the suspension fluid in which more freshly washed frog's muscle was allowed to respire in a Barcroft micro-respirometer, by means of which its oxygen uptake was measured. Some more of the same "Koch-saft" was stored at 0° out of contact with the air for the same period of time and its effect on the respiration of washed frog's muscle was compared with that of the aerated "Koch-saft."

If the "respiration-substance" were a true co-enzyme and the oxidisable substances were contained in the washed muscle the velocity of respiration in the two cases should not differ considerably. If, however, the oxidisable substances were contained in the "Koch-saft" the unused one should be the more active. In the latter case the existence of a respiration co-enzyme would not be disproved. The assumption of its existence would, however, seem to be unnecessary.

Experimental details.

The yeast extracts were made by boiling some baker's yeast with about twice its weight of distilled water for a few minutes and then filtering as quickly as possible. They were adjusted with caustic soda to p_H 7·6, and divided into two portions. One was passed through a Berkefeld filter and stored in small evacuated tubes in an ice-chest. The other portion was aerated at room temperature in contact with some frog's muscle which had been washed several times with distilled water. The exact figures for each case are given in the table of results. It was then filtered from the muscle, passed through a Berkefeld filter and stored in an ice-chest until it was used. In order to determine the effects of the two portions on the respiration of washed frog's muscle some more of the latter was taken and half a gram weighed out into each of three respirometers. In them were placed 3 cc. respectively of each of the two samples of "Koch-saft" and of a 1 % solution of KH_2PO_4, each being adjusted with caustic soda to p_H 7·6. They were placed in a bath maintained at a known temperature and shaken by means of an electric motor. Soda was placed in the cups to absorb the carbon dioxide evolved.

In experiments 7 and 8, to the yeast extract was added an excess of saturated lead acetate solution and enough of a suspension of lead hydroxide to keep the liquid slightly alkaline. This removes a large number of substances, among them glutathione and any inorganic phosphates. The precipitate was filtered off and the excess lead was removed from the filtrate with sulphuretted hydrogen and the solution concentrated *in vacuo*. The acid potassium phosphate was added in experiment 8 in accordance with the discovery by Meyerhof that it makes the respiration velocity more nearly constant during the experiment.

It can be seen from the table of results that during respiration a great part of the "respiration-substance" is destroyed.

		1st respiration					2nd respiration					
								cmm. O_2 absorbed per gram of muscle in one hour			Frog's muscle	
No.	Extract	Vol. cc.	Wt. of frog's muscle grams	Times washed	Vols. of H_2O each washing	Time of aeration hours	Temp. °C.	(1) In phosphate	(2) In previously respired extract	(3) In stored extract	Times washed	Vols. of H_2O each washing
1	Yeast	30	2·5	6	50	18	23	36	98	424	7	50
2	,,	45	4·5	8	50	18	22	42	88	254	8	75
3	,,	30	3	6	60	18	23	20	20	296	8	50
4	,,	26	2·6	8	50	21	22	30	33	296	8	50
5	,,	50	5	6	50	23	22	33	147	420	8	80
6	,,	50	5	6	50	45	21·5	37	116	337	8	80
7	Yeast filtrate after basic lead acetate	50	5	6	50	44	23·5	43	58	558	8	60
8	As 7 but with 1 % KH_2PO_4 added	40	4	6	50	20	21·5	28	105	449	8	75

Discussion of Results.

Meyerhof observed that the addition of yeast or of tissue extracts to washed tissue increases the velocity of its respiration. He attributed this effect to the presence in such extracts of a co-enzyme which, working in conjunction with the washed tissue, increased the velocity of the oxidation of the oxidisable substances present. It seemed to him that the known oxidisable substances were present in too small a concentration to account for the effects observed.

Research, subsequent to the publication of Meyerhof's papers, has extended our knowledge of the number and concentration of oxidisable substances contained in such extracts. This knowledge, in conjunction with the results described in this paper, suggests that the stimulation of the respiration of washed tissues, observed on the addition of such extracts, may reasonably be attributed to the united effects of the different oxidisable substances therein contained, rather than to the presence of a co-enzyme, the necessity for which whether as an oxygen-carrier or in any other capacity has not yet been clearly demonstrated.

Summary.

If an extract of yeast, made with boiling water, be aerated for some hours at room temperature in contact with some washed frog's muscle it loses its power to increase the velocity of oxygen uptake of another preparation of fresh washed muscle. This suggests that the effects attributed to a "respiration-substance" capable of providing a general stimulus to intracellular oxidative processes are really to be attributed to the collection of oxidisable substances contained in the yeast extract. When these have been oxidised the extract has no further power to stimulate the respiration of washed frog's muscle.

REFERENCES.

Batelli and Stern (1911). *Biochem. Z.* **33**, 314.
Harden and Young (1906). *Proc. Roy. Soc.* **77**, 405.
Meyerhof (1918). *Pflüger's Arch.* **170**, 367.
—— (1919, 1). *Pflüger's Arch.* **175**, 20.
—— (1919, 2). *Z. physiol. Chem.* **101**, 172.

XLX. THE EFFECT OF AIR, WHICH HAS BEEN EXPOSED TO THE RADIATIONS OF THE MERCURY-VAPOUR QUARTZ LAMP, IN PROMOTING THE GROWTH OF RATS, FED ON A DIET DEFICIENT IN FAT-SOLUBLE VITAMINS.

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A Report to the Medical Research Council.

(*Received March 7th, 1923.*)

Introductory.

It has already been demonstrated [Hume, 1922; see also Goldblatt and Soames, 1922] that young rats fed on a diet deficient in fat-soluble vitamins, grow normally for a much longer period, when they are irradiated with the mercury-vapour quartz lamp, than when not so treated. Experiments by Kestner [1921, 1922], to which attention was directed by Professor Hans Meyer, Vienna, suggested the possibility that exposure to a source of ultra-violet light might not be the only means of thus inducing growth, but that exposure to air through which such light had passed might also be effective. Kestner sought to disentangle the factors which give to a mountain climate its well-known health promoting properties. It had already been observed [Laquer, 1913, 1919; Weber, 1919] that in dogs rendered anaemic, the red blood corpuscles are regenerated more quickly at an altitude (Monte Rosa) than in the plains, an effect generally attributed to the reduced oxygen pressure at high altitudes. Kestner made his tests with dogs rendered anaemic by bleeding or by injection of pyrodin, and he observed the rate of regeneration of red cells in control animals and in those under the influence of (*a*) reduced atmospheric pressure, (*b*) the light from a naked carbon arc lamp, and (*c*) the air drawn from the neighbourhood of such a lamp. Reduced pressure produced no more rapid regeneration than occurred in control animals, but the direct rays of the lamp or air drawn from the neighbourhood of the lighted lamp both caused the regeneration to be more rapid and complete. It seemed therefore worth while to test whether air which had been

exposed to a source of ultra-violet light, also possessed the power of prolonging normal growth in rats fed on a diet deficient in fat-soluble vitamins. This was tested by means of the following experiments. Experiment 1 was performed in Vienna and experiments 2–4 at the Lister Institute.

The diet deficient in fat-soluble vitamins had the following composition: heated caseinogen, 180 g.; maize starch, 520 g.; hardened cotton-seed oil, 150 g.; salt mixture, 50 g.; marmite (yeast extract), 60 g.; lemon-juice, 50 g.; water, 600 g. The salt mixture had the following composition: sodium chloride, 51·9 g.; mag. sulph., 164·0 g.; sodium acid phosph., 104·1 g.; potassium phosph. 286·2 g.; calcium phosph., 162·0 g.; calc. lact., 390·0 g.; ferric citrate, 35·4 g.

All rats, both treated and controls, were placed upon the deficient diet at a weight of about 50 g., and treatment, where such was instituted, began from that time or a few days later. Wherever possible controls were taken from the same litter.

The mercury-vapour quartz lamp used for the Vienna experiments was of the Hanau pattern; it was always allowed to run for at least five minutes before being used. No estimate was made of its deterioration since purchase, but this was probably great. The lamp used for the Lister Institute experiments was a 74 pistolette quartz lamp made by the Hewittic Electric Company, using a current of 3·5 ampères and emitting rays whose shortest wave length was 230μ.

The treated rats and their controls were kept, each animal separately, in 8-litre glass cylinders, with sawdust at the bottom. The treatment with irradiated air was carried out as follows. Every second day, the rats were removed from the glass jars and left in the animal room, while the jars were conveyed to the room containing the mercury-vapour lamp, which in the Kinder Klinik was at the extreme other end of the building, and, in the Lister Institute, on another floor. The jars were placed below the lamp, with their bases about 80 centimetres distant from it, so that the light shone straight down into them. They remained in this position for ten minutes, were then covered with glass lids and conveyed back to the animal room. The animals were put in and the covers replaced to hinder diffusion of the air. *After ten minutes* the covers were removed and not replaced but the animals continued to live in the jars until the next treatment.

Experiment 1. Three animals, all from the same litter of three, were treated with irradiated air. One had been for seven days and the other two for four days, on the deficient diet, before treatment began. The weight curves, however had not begun to flatten off and even if they had done so, previous experiment [Hume, 1922] has shown that for a considerable time after being placed on the deficient diet, rats are capable of responding to the stimulus of radiation and resuming normal growth.

Control animals from the same litter were not available but other rats bred in the same way and placed upon the deficient diet a little previously,

were used for that purpose. These animals were also used as controls for a group of rats, irradiated directly with the mercury-vapour lamp, of which the details have already been published [Hume, 1922, Chart 1]. The curves of both these groups are reproduced here for comparison.

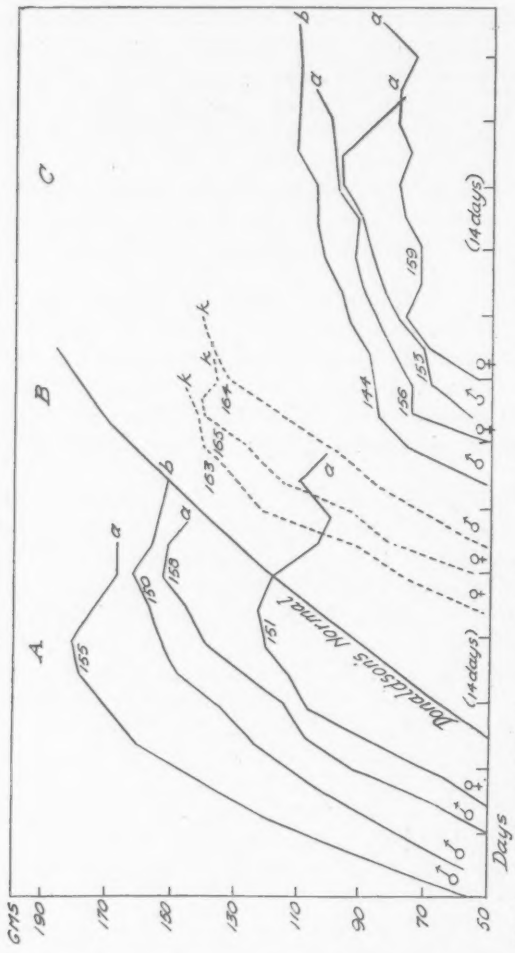

Chart 1. *Experiment* 1. Weight curves of rats fed on a diet deficient in fat-soluble vitamins.
A. Irradiated directly, with the mercury-vapour quartz lamp, for ten minutes every second day.
B. Treated with air, exposed to the mercury-vapour quartz lamp in glass jars for ten minutes every second day.
C. Untreated controls.
(Members of the same litter are designated by the same small letter.)

Chart 1 shows the weight curves of (*A*) four rats on "−A" diet, radiated directly for ten minutes every second day, (*B*) the three rats treated with irradiated air and (*C*) four untreated controls. Curves marked with the same small letter belong to animals of the same litter. It is at once apparent that the growth of the rats treated with irradiated air is nearly, though not quite,

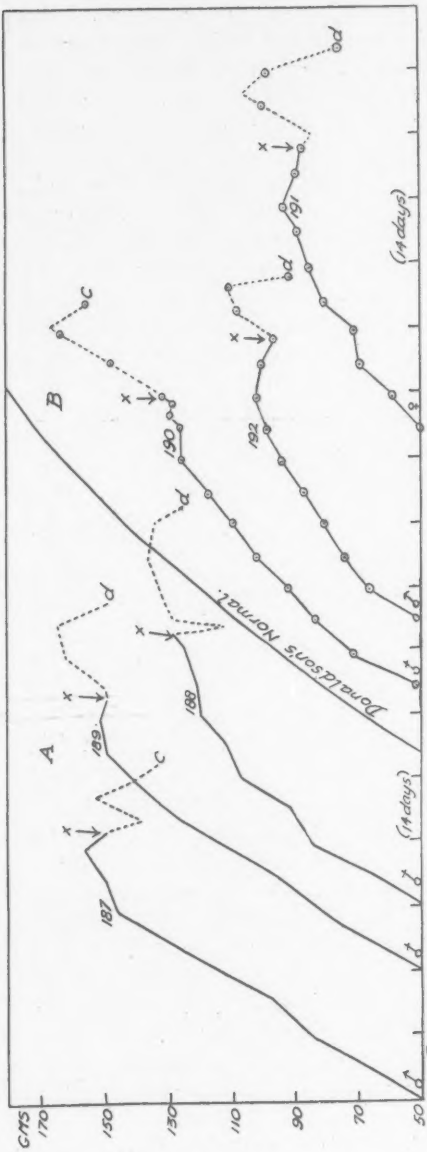

Chart 2. Experiment 2. Weight curves of rats fed on a diet deficient in fat-soluble vitamins.
A. Treated with air, exposed to the mercury-vapour quartz lamp in glass jars for ten minutes every second day.
B. Untreated controls.
From X, the animals exposed directly to the rays of the lamp, for ten minutes daily.
(Members of the same litter are designated by the same small letter.)

as good as that of the directly radiated animals and is much better than that of the untreated controls. In the controls normal growth continued for a week, in the rats treated with irradiated air it continued for four weeks, and in the animals directly irradiated for from 3–7 weeks.

Experiment 2, *Chart* 2. This experiment was similar to experiment 1. Two rats from one litter, and four rats from another litter were divided into two groups of three, so that the two litters were equally represented in each group. One group of three, Chart 2 (*A*), was treated with irradiated air, and the other group of three served as controls, Chart 2 (*B*). The same superiority of the treated rats (*A*) over the untreated controls (*B*) is apparent, as in experiment 1. The controls in this case are slightly more strongly growing and the growth of the strongest control is as good as that of the weakest treated rat. Reference to the previous papers of Hume and of Goldblatt and Soames, shows, however, that this may also occur when rats are irradiated directly.

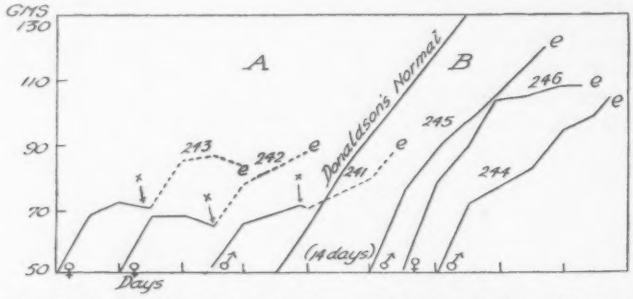

Chart 3. *Experiment* 3. Weight curves of rats fed on a diet deficient in fat-soluble vitamins.

A. Treated for one hour daily, with air drawn from the neighbourhood of the mercury-vapour quartz lamp. From X, treatment changed, animals treated with irradiated air in jars.

B. Untreated controls.

(Members of the same litter are designated by the same small letter.)

On the 60th day of experiment, when growth had practically ceased in every case all the six rats were exposed directly to the rays of the lamp for ten minutes every day. All made a small response and resumed growth for a period, which, except in the case of one control, was only of very short duration. The response of the controls was however the greater.

A dose of freshly irradiated air, given every second day, did not therefore evoke quite the maximum growth response, capable of being produced by direct irradiation of the animal, but it did produce a very marked prolongation of normal growth, beyond that shown by controls. A dose of irradiated air, given more often than once every second day, might be able to produce the maximum response.

Experiment 3, *Chart* 3. In this case the rats were exposed to air, drawn from the neighbourhood of the lighted mercury-vapour lamp, the animals being shielded from the illumination from the lamp.

A 4·5 ampère Hanau lamp was used. The lamp was surrounded by an iron shield. This iron shield which was earthed by a wire to the gas pipe was fairly air-tight, except for the ventilation holes above and below and the opening in front for the illumination to pass. The upper ventilation holes were closed with corks and a tinned funnel (10 cm. diameter) was soldered over the illumination aperture. To the stem of the funnel, 11 feet (3·3 metres) of glass tubing of 0·8 cm. outer bore, was attached, the other end of which passed through a light-tight box. The lamp was kept cool by a wet bandage around the shield.

The rats were placed singly in large bottles enclosed in a light-proof box. The bottles were closed with rubber bungs, admitting inlet and outlet tubes. Air was sucked through the whole system by a water pump. The ventilation was at the rate of 20 litres per hour. Ozone was produced in the neighbourhood of the lamp and could be detected in the bottles and at the outlet; the amount diminished as the distance from the lamp increased. After the lamp had been lit for 15 minutes, the box containing bottles and rats was brought into the room, joined up and left there for one hour daily.

Six rats were used for the experiment, all belonging to the same litter. Three were exposed to the air from the lamp and three were left untreated and used as controls.

Chart 3 shows the weight curves of the three treated rats (*A*) and of the three controls (*B*). After 20 days it was apparent that normal growth would not be prolonged by treatment with irradiated air in this way; the growth of the treated rats was inferior even to that of the controls, which in this instance showed remarkably strong growth. On the 20th day treatment with air drawn from the lamp was stopped, and treatment in jars of radiated air, as described under experiment 1, was instituted. The jars were freshly irradiated every day for ten minutes. This treatment was continued for 20 days and a moderate growth response was obtained (see Chart 3 (*A*), curves from X onwards).

It was therefore concluded that air drawn from the neighbourhood of the lamp, by means of such an apparatus as that described, does not possess the power of prolonging normal growth in rats, fed on a diet deficient in fat-soluble vitamins. The inferiority in the growth of the rats treated, to that of the controls, suggests that some factor, actually detrimental, was at work; this may have been the ozone. At any rate it seems safe to conclude that the ozone is not the growth stimulating factor.

Experiment 4, *Chart* 4. This experiment was a repetition of Nos. 1 and 2. In addition an extra series of jars was irradiated in the same way, but the air was blown out and fresh, not irradiated, air was admitted before the rats were placed in the jars. The jars were blown out with 20 strokes of a bellows, a process occupying a very short time, and only delaying the entrance of the rats into the jars by about 40 seconds. All jars were irradiated every day, instead of every second day as in experiment 1. Four litters of rats were used

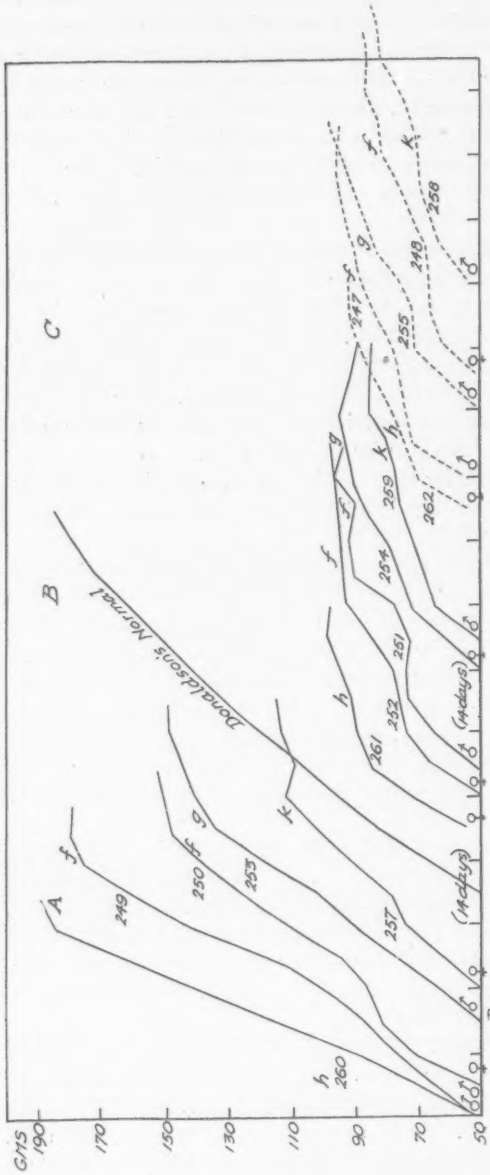

Chart 4. *Experiment* 4. Weight curves of rats fed on a diet deficient in fat-soluble vitamins.

A. Treated in irradiated jars containing irradiated air.

B. Treated in irradiated jars containing air not irradiated.

C. Untreated controls.

(Members of the same litter are designated by the same small letter.)

and each litter was divided equally between the three experimental groups, *i.e.* (*A*) treatment in irradiated jars, containing irradiated air, (*B*) treatment in irradiated jars containing air, not irradiated, and (*C*) control animals undergoing no treatment. Each group contained five animals. Chart 4 shows the weight curves of the three groups. The result is quite unequivocal; the rats treated with irradiated air (*A*) show prolongation of normal growth, while those occupying irradiated jars from which the irradiated air has been displaced, (*B*) behave like the untreated controls (*C*).

It is therefore evident that the growth promoting property lies in the irradiated air itself, and not in any property which might have been acquired by the glass.

SUMMARY.

(1) Rats fed on a diet deficient in fat-soluble vitamins, when kept in glass jars which had been exposed to the mercury-vapour quartz lamp for ten minutes every second day, showed prolongation of normal growth over controls not so treated.

(2) Rats exposed to the air containing ozone drawn over the quartz lamps and passed through 3 metres of glass tubing, 0·8 cm. bore, showed poorer growth than the control animals. The same rats, subsequently treated in glass jars of irradiated air gave some growth response.

(3) Rats placed in irradiated jars from which the irradiated air had been displaced, showed no prolongation of normal growth.

These results are analogous to the accelerated regeneration of red blood corpuscles in anaemic dogs observed by Kestner. In both cases the effect was obtained by exposure of the animals to air through which ultra-violet light has passed, as well as by exposure to the light itself. Kestner used a 40 ampère naked carbon arc lamp and the air was drawn from the neighbourhood of the lamp and passed through a box in an adjoining room in which the dogs were placed. The animals were supplied with this air for 3 to 5 hours daily.

Kestner concludes that as a result of sun radiation or radiation from the carbon arc lamp, substances are formed which, if breathed, stimulate the formation of blood corpuscles. He further suggests the possibility that compounds of nitric oxide, formed when the lamp is alight, are responsible for the action.

In the present experiments, a mercury-vapour lamp enclosed in quartz was used. This is not believed to produce nitric oxide. In experiment 3 the air drawn away from the lamp was found to possess no growth-promoting activity, but it may have been exposed for too short a time to the lamp or the character of the apparatus used may have been such as to deprive it of this activity before it could reach the rats. The negative result obtained in experiment 4 when the irradiated air in the jars was displaced makes it plain

that it is *the air and not some property impressed on the glass jars which is active.*

Ozone was present in the air in experiment 3 which gave a negative result; it is therefore clear that ozone is not responsible.

The other effect produced on air by ultra-violet radiation is a small degree of ionisation, a change which disappears rapidly. Air ionised in capacious jars, by reason of the large volume and less relative surface exposed to glass, would remain ionised somewhat longer than when drawn through glass tubes, with rubber junctions. This may explain the positive result obtained with the former method and the negative one obtained with the latter.

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REFERENCES.

Goldblatt and Soames (1922). *Lancet*, ii. 1321.
Hume (1922). *Lancet*, ii. 1318.
Kestner (1921). *Z. Biol.* **73**, 1, 7.
—— (1922). *Beiträge zur Klinik der Tuberkulose*, **50**, 121.
Laquer (1913). *Deutsch. Arch. klin. Med.* **110**, 189.
—— (1919). *Z. Biol.* **70**, 118.
Weber (1919). *Z. Biol.* **70**, 131.